The role of STAT4 transcription factor in purified human Dendritic cells treated with interferon-beta, an autoimmune disease model

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การศึกษาบทบาทของแสตตสี่ในเซลล์เม็ดเลือดขาวชนิดเดนไดร์ตที่ถูกกระตุ้นด้วยอินเตอร์เฟียรอนชนิดเบต้าแบบจำลองในโรคภูมิแพ้ตนเอง

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เทอดเกียรติ ผลบุญ : การศึกษาบทบาทของแสตตสี่ในเซลล์เม็ดเลือดขาวชนิดเดนไดร์ตที่ถูกกระตุ้นด้วยอินเตอร์เฟียรอนชนิดเบต้าแบบจำลองในโรคภูมิแพ้ตนเอง. (The role of STAT4 transcription factor in purified human Dendritic cells treated with interferon-beta, an autoimmune disease model) อ.ที่ปรึกษาหลัก : ดร.ภัทริน ตั้งธนตระกูล, อ.ที่ปรึกษาร่วม : ดร. พิมพเยาว์ สดใส

SNPs ของ STAT4 มีความเกี่ยวข้องกับการเกิดโรคภูมิแพ้ตนเองชนิดต่างๆ แม้ว่าจะมีบทบาทสำคัญในการกระตุ้นการสร้าง IFN-γ ในเซลล์เม็ดเลือดขาวหลายชนิด แต่ข้อมูลเกี่ยวกับการแสดงออกและหน้าที่ของ STAT4 ใน dendritic cell (DC) แต่ละชนิดนั้นยังมีอยู่อย่างจำกัด ดังนั้นเพื่อต้องการศึกษาการแสดงออกและบทบาทของ STAT4 ใน DC ผู้วิจัยได้ทำการแยก DC จากผู้บริจาคที่มีสุขภาพดีจำนวน 6 ราย ด้วยวิธี magnetic isolation ตามด้วย flow cytometry ในการแยก dendritic cell ออกเป็น 3 ชนิดย่อย คือ plasmacytoid dendritic cell (pDC; $CD123^{+}$), conventional dendritic cell 1 (cDC1; $CLEC9A^{+}$), และ conventional dendritic cell 2/3 (cDC2/3; $CD1c^{+}$) หลังจากนั้นจะนำ DC ที่ได้ไปกระตุ้นด้วย IFN-β เพื่อวัดการแสดงออกของยีน *STAT4* ด้วย Real-Time PCR และ โปรตีน STAT4 ด้วย Western blotting โดย DC ที่มีการแสดงออกของ STAT4 จะถูกนำมาศึกษาบทบาทด้วยวิธี lisofylline inhibition และวัดการหลั่ง Cytokine หลังถูกกระตุ้น จากผลการทดลองพบว่าจำนวน pDC, cDC1, และ cDC2/3 ในผู้บริจาคมีค่าเฉลี่ยประมาณ 0.07%, 0.04% และ 0.31% เป็นที่น่าสนใจว่าเมื่อนำ DC มากระตุ้นด้วย IFN-β แล้วมีเพียง cDC2/3 เท่านั้นที่มีการแสดงออกของยีน *STAT4* และโปรตีน pSTAT4 เพิ่มขึ้นอย่างมีนัยสำคัญ และส่งผลให้มีการหลั่ง IFN-α2 สูงขึ้นอย่างมีนัยสำคัญ ยิ่งไปกว่านั้น Lisofylline ยังไม่สารยับยั้งการถูกกระตุ้นของโปรตีน pSTAT4 จากการศึกษานี้ได้แสดงให้เห็นว่า STAT4 อาจมีบทบาทที่สำคัญในการควบคุมการตอบสนองของ DC ในสภาวะที่ถูกกระตุ้นให้เกิดการอักเสบได้

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STAT4 (Signal Transducer Activator of Transcription factor 4) polymorphisms are risk factors contributing to autoimmune disease. While STAT4 is dominant in promoting interferon-γ in T lymphocytes and NK cells, its existence and function in dendritic cells (DCs) are still absent. To characterize the STAT4 in human DCs, we isolated dendritic cells from buffy coat of healthy blood donors (n = 6). The dendritic cells are enriched and specifically sorted into 3 subtypes including plasmacytoid dendritic cells (pDCs, $CD123^+$), $CD8^+$ priming dendritic cells (cDC1, $CLEC9A^+$), and $CD4^+$ priming dendritic cells (cDC2/3, $CD1c^+$) using magnetic separation and flow cytometry. By quantitative PCR and western blotting, the presence of *STAT4* gene expression as well as phosphorylated STAT4 was determined in both IFN-β-induced dendritic cell maturation and immature dendritic cells *ex vivo*. In addition, we study the role of STAT4 by performing lisofylline inhibition and cytokine secretion. Regarding our analysis, the percentages of each dendritic cell subset are 0.07% for pDC, 0.04% for cDC1, and 0.31% for cDC2/3 in healthy blood donors. The IFN-β-enhanced *STAT4* expression and phosphorylated STAT4 were prominent in mature dendritic cells, especially in cDC2/3, but not in pDC and cDC1 (p-value < 0.01). As cDC1 and cDC2/3 are responsible for T-cell priming, this indicated that IFN-β-induced STAT4 might be crucial in regulating dendritic cells producing IFN-γ. In addition, STAT4 and pSTAT4 were not inhibited by lisofylline pretreatment. Lastly, we found that the secretion of IFN-α2 was increased in cDC2/3 after IFN-β stimulation. Our study highlights the significance of STAT4 in dendritic cells which might be involved in the IFN-mediated autoimmune diseases.

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Literature review

1. Literature review

1.1 Type-I interferon

The Type-I interferon (IFN-I) family comprises interferon-α (IFN-α) and interferon-β (IFN-β). These two types differ in their sources within the body. IFN-α is primarily produced by white blood cells and cells involved in blood cell formation (hematopoietic cells). On the other hand, IFN-β is generated by fibroblast cells and cells lining the surfaces of organs (epithelial cells) (1). These interferons function through a signaling pathway known as JAK-STAT. This pathway involves various STAT proteins that respond differently to the different interferon types. The way they trigger responses depends on how well they are recognized by two subunits of the IFNAR receptor, which is linked to the specific type of IFN-I molecule (2).

When the body encounters intracellular threats like viruses or certain bacteria (such as gonococcal bacteria), it produces Type-I interferons as a defensive measure (3). These interferons induce a protective antiviral state in nearby cells, shielding them from potential viral infiltration. Interestingly, Type-I interferons also emerge in response to components from the body's own cells, like self-nucleic acids, self-proteins, and even fragments released from dying cells (4). Once released, these interferons activate and draw immune cells to the site. This process sets in motion a series of events that influence the immune system, triggering local inflammation that can potentially extend to a broader systemic inflammation.

In the context of systemic autoimmune diseases (SADs) such as Systemic Lupus Erythematosus (SLE), the primary cause of excessive Type-I IFN production arises from the inadequate removal of apoptotic materials. This leads to the activation of plasmacytoid dendritic cells (pDC), which are the main source of Type-I interferon in this scenario (4). These interferons also contribute to the development of dendritic cells (DC), which are crucial for the maturation and movement of immune cells. Additionally, Type-I interferons facilitate the priming of T cells, stimulate changes in B-cell function (called isotype switching), and support the creation of humoral immunity (5). While the extent to which an increased rate of apoptosis occurs remains uncertain, earlier research suggests that this occurrence could be linked to impaired functioning of phagocytic cells, particularly monocytes (4). Furthermore, prior studies indicate that IFN-β can promote the maturation of cDC3, a type of dendritic cell that plays a significant

role in the development of SLE. Despite this insight, there is still a lack of understanding about the connection between IFN-β and SLE (6).

In contrast to SAD, IFN-β in the context of multiple sclerosis unveils a multifaceted impact on the immune system. Specifically, IFN-β reduces the concentrations of cDC within peripheral blood. This reduction extends to alterations in the functional DCs and other APCs, resulting in a downregulation of antigen presentation and a modulation of APCs' capacity to incite T-cell responses (7). This regulatory influence of IFN-β appears to modulate the immune activities, introducing a calibrated modulation that tempers certain immune responses while simultaneously preserving the crucial population of IFN-I secreting pDCs (8). In addition IFN-β exhibits a dual effect on Toll-like receptor 9 (TLR9) expressed on pDCs. On the other hand, IFN-β downregulates TLR9, leading to the reduction in TLR9-mediated secretion of Th1-promoting IFN-α from pDCs (9). This refined regulatory role of IFN-β unfolds the additional actions on pDCs, it enhances the expression of Toll-like receptors 3 and 7 (TLR3, TLR7), alongside the pivotal adaptor molecule MyD88. This upregulation is postulated to contribute to increased immune regulation, presenting IFN-β as a central player in orchestrating a balanced and finely tuned immune response in the intricate landscape of multiple sclerosis but not in SLE (10).

In conclusion, Type-I interferons, specifically IFN-α and IFN-β, serve as vital components of the immune response against both external and internal threats. Their intricate interactions with immune cells, such as dendritic cells, T cells, and B cells, contribute to the body's defense mechanisms and play a role in the development of autoimmune disorders like SLE. Further investigation into the relationship between IFN-β and SLE could provide valuable insights into disease mechanisms and potential therapeutic strategies.

1.2 Systemic lupus erythematosus and interferon type I

Systemic Lupus Erythematosus (SLE) is characterized by a notable increase in the presence of Type-I interferon (IFN-I) in the bloodstream as a response to the body's own substances, causing widespread inflammation throughout the body (11). An intriguing occurrence has been documented where individuals receiving IFN-α therapy for the treatment of malignant carcinoid tumors developed symptoms resembling SLE, such as high levels of anti-dsDNA autoantibodies, muscle pain (myalgia), and inflammation in multiple joints (arthritis). These SLE-like symptoms vanished upon discontinuation of IFN-α therapy (12).

In recent times, a groundbreaking discovery has been the development of anifrolumab, a specialized antibody designed to obstruct the interferon-α receptor. This innovation has undergone clinical trials as a potential treatment for SLE. Impressively, it has demonstrated positive outcomes among patients whose skin is affected by the disease. However, it's worth noting that there have been reports of an increased risk of infection from the herpes simplex virus in these patients (13).

To delve deeper into understanding SLE, scientists have performed transcriptomic analyses. One of these studies unveiled a significant increase in the activity of genes related to the interferon response, known as interferon signature genes (ISGs), in individuals with SLE (14). Consequently, Type-I interferon and ISGs have been established as hallmarks of SLE, representing a key source of the systemic inflammation characteristic of the disease.

While targeting the IFN-I receptor presents a promising avenue for controlling the autoimmune processes underlying SLE, it's important to acknowledge that dampening these signals can heighten the vulnerability to infections. This intricate balance between therapeutic benefits and potential drawbacks highlights the need for more precise approaches in therapy development. By narrowing down the focus to specific therapeutic targets, it becomes feasible to mitigate undesirable side effects while still effectively modulating autoimmune mechanisms. This underscores the ongoing quest for therapies that strike the optimal equilibrium between immune regulation and infection prevention.

1.3 Dendritic cells and its role in SLE

The DCs subtypes and cellular markers has been summarized in Table 1 (15, 16). Among these, a recent discovery has unveiled a novel subset known as cDC3s, prompted by stimulation from IFN-β. These cDC3s stand out due to their expression of GITRL, a specific marker (6). Both cDC2s and cDC3s play crucial roles in activating and transforming naive T cells into mature ones. However, cDC3s possess a heightened ability to incite Th17 responses, known for their proinflammatory nature, thus exhibiting an augmented proinflammatory profile in human systems (17).

The intricate chain of events in the development of systemic lupus erythematosus (SLE) can be traced back to the impediment in the efficient clearance of dying cells and the continual release of cellular components like DNA, RNA, and proteins, which are persistently found in SLE patients (18). These self-antigens can set off plasmacytoid dendritic cells (pDCs) to increase their

production of Type-I interferon (IFN-I), which constitutes a major trigger for systemic inflammation. The secreted IFN-I can perform a dual role—it can activate self-reactive T-cells, instigating the B-cell response via a T-cell dependent mechanism (19). Simultaneously, it can also fuel the production of auto-antibodies, thereby initiating a feedback loop involving conventional dendritic cells (cDCs). This loop enhances their proficiency in presenting self-antigens to T-helper cells, thus perpetuating a cycle of inflammation (19) (Figure. 1).

Numerous past investigations have proposed that the accumulation of dendritic cells within organs is closely intertwined with tissue inflammation and the progression of SLE. This is substantiated by the decrease in the count of circulating pDCs and cDCs, which corresponds to disease activity in individuals with active SLE (20, 21). Additionally, another noteworthy function of Type-I interferon (IFN-I) is its role in guiding the differentiation of Th1 cells to produce IFN-γ, a proinflammatory cytokine, along with the stimulation of Th17 responses. While the exact mechanism behind IFN-I-driven Th17 induction remains elusive, evidence does suggest a correlation between elevated IFN-I levels and an increased number of circulating Th17 cells in SLE patients (22, 23).

Table 1 Type of dendritic cells with their surface markers in human, main source, and role in autoimmune pathogenesis (15, 16)

	Plasmacytoid DCs	**cDC1**	**cDC2**	**cDC3**	**moDC**
Markers in human	CLEC4C (BDCA2), CD123, CD11c low/neg	CD141 (BDCA3), XCR1, CLEC9A	CD1c (BDCA1) with $CD5^+$	CD1c (BDCA1) with $CD5^-$	CD14, MR (CD206)
Main source	Bone marrow and peripheral lymphoid tissues	Lymph nodes and peripheral tissues	Spleen and peripheral tissues	Spleen and peripheral tissues	Rare in steady state, lymphoid and peripheral tissues in inflammation state
Role in autoimmune pathogenesis	Major source of type I IFN production	Activation of $CD8^+$ T cells through cross-presentation	proliferation of pathogenic $CD4^+$ T cells through presentation	Presenting the antigens to $CD4^+$ T-cells and $CD8^+$ T-cell, Induce Th17 maturation	Unclear

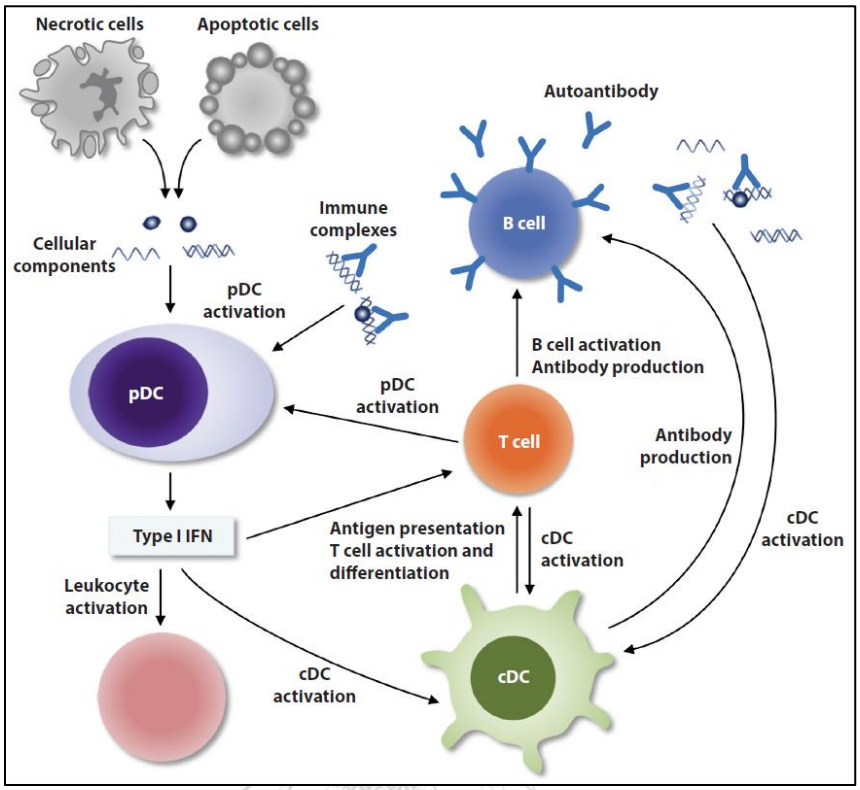


Figure 1 The role of SLE developed by the dendritic cells (19).

The role of SLE developed by the DC start with cellular components or immune complexes activate the pDC to secreted type-I IFN. Then the T-cells are polarized and affect to activate B-cell to produce autoantibodies against self-antigens and cDC to increase self-antigen processing and presentation

1.4 Signal transducer and activator of transcription 4 (STAT4) proteins

A recent Genome-Wide Association Study (GWAS) has shed light on three potential candidate genes within the Type-I interferon signaling pathway. These genes include interferon regulatory factor 5 (IRF5), tyrosine kinase 2 (TYK2), and signal transducers and activators of transcription factor 4 (STAT4) (24). STAT4, in particular, has been linked to an increased susceptibility to systemic lupus erythematosus (SLE) and a more severe disease manifestation (25). Interestingly, the influence of STAT4 extends beyond SLE, as it has also been implicated in other autoimmune conditions like rheumatoid arthritis, type-I diabetes, psoriasis, and systemic sclerosis (26). An experimental approach using STAT4 antisense oligonucleotide (STAT4 ASO) in lupus nephritis mice resulted in a reduction of disease severity (27). However, a separate study involving SLE-afflicted mice proposed that STAT4 within B-cells doesn't significantly impact autoantibody production or the deposition of immune complexes in the kidney (2). These

divergent findings emphasize the significance of further investigating the role of STAT4 in deciphering the true underpinnings of autoimmune diseases. Moreover, this exploration could serve as a basis for identifying potential novel therapeutic targets in the future.

The family of Signal Transducer and Activator of Transcription (STAT) proteins plays a pivotal role in gene transcription activation in response to various cytokines, including Type-I interferons, IL-12, IL-23, IL-2, IL-27, and IL-35 (Figure. 2) (28, 29). The STAT activation begins with the interaction between the cytokines and their receptor, then the phosphorylation is induced at the key tyrosine residue in the STAT transactivation domain by Janus kinases (JAKs), SRC family kinases, and other tyrosine kinases. This leads to STAT-STAT dimerization, nuclear translocation, DNA binding, and the transcriptional induction of genes in the nucleus (Fig.2). The transcriptional outcomes by differing STAT protein give the various mRNA which translates to many proteins that could have a variety of cellular effects, in contrast, if any activated STAT protein is impaired, this would lead to the development of many diseases especially STAT4 which has been accepted that highly correlated to SLE (30).

Comprising 7 members—STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6—these proteins are induced by diverse cytokines via the JAK-STAT pathway (Figure. 3). They serve as regulatory entities governing a wide range of physiological and pathological processes, from immune responses to cytokine release (31, 32). In addition to differences in function, inducers, and targeted genes, variations in structural features are also evident across the STAT family. The structure of STAT proteins encompasses 6 domains (Figure. 4), each with its distinct role:

1. N-terminal domain: dimerizes inactivated STATs and promotes nuclear translocation.

2. helical coiled coil: provides a carbonized hydrophilic surface and binds to regulatory factors

3. DNA-binding domain: binds to an enhancer of the GAS family

4. linker domain: involves in the DNA binding process

5. Src homology (SH2) domain: binds specifically to the cytokine receptor after tyrosine phosphorylation

6. C-terminal transactivation domain: activates transcriptional process (33)

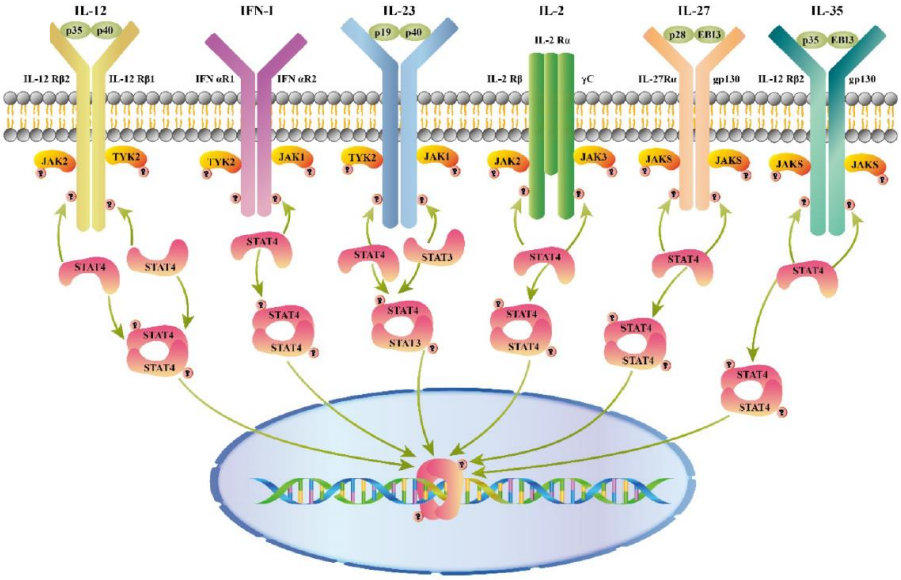


Figure 2 Cytokines Triggering STAT4 Activation and STAT4 signaling pathway *(29)*

Cytokines and their receptor complexes involved in initiating STAT4 signaling. Key players include JAK (Janus kinase), STAT (Signaling transducer and activator of transcription), and TYK (Tyrosine kinase). STAT4 becomes phosphorylated upon binding of diverse cytokines (IL12, IFN-I, IL23, IL2, IL27, IL35) to the cell membrane. The activated and dimerized STAT4 molecules then migrate to the cell nucleus to orchestrate gene expression regulation (29).

STAT	Cellular functions	Major diseases
1	• Cell growth and apoptosis • T_H1 cell-specific cytokine production • Antimicrobial defence	• Atherosclerosis • Infection • Immune disorders
2	• Mediation of IFNα/IFNβ signalling	• Cancer • Infection • Immune disorders
3	• Cell proliferation and survival • Inflammation • Immune response • Embryonic development • Cell motility	• Cancer
4	• T_H1 cell differentiation • Inflammatory responses • Cell proliferation	• Experimental autoimmune encephalomyelitis (multiple sclerosis) • Systemic lupus erythematosus
5A	• Cell proliferation and survival • IL-2Rα expression in T lymphocytes • Mammary gland development • Lactogenic signalling	• Cancer • Chronic myelogenous leukaemia
5B	• Cell proliferation and survival • IL-2Rα expression in T lymphocytes • Sexual dimorphism of body growth rate • NK cell cytolytic activity	• Cancer • Chronic myelogenous leukaemia
6	• Inflammatory and allergic immune response • B cell and T cell proliferation • T_H2 cell differentiation	• Asthma • Allergy

Figure 3 Role of STAT protein in cellular functions relating to the disease *(30)*

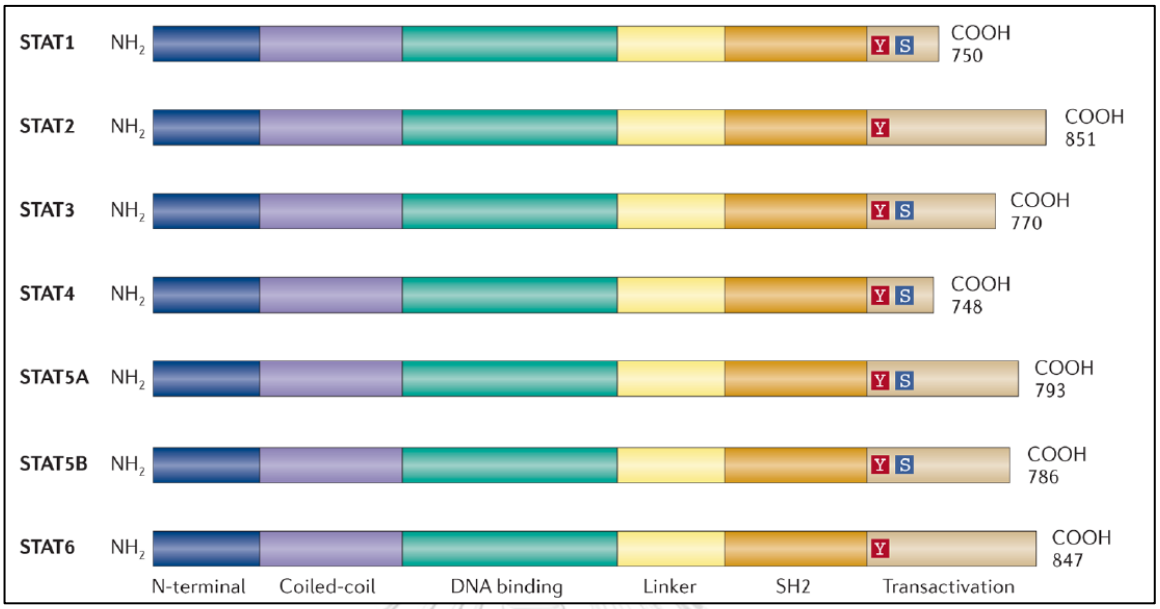


Figure 4 Structure of STAT proteins (30)

Diagram showing the linear arrangements of domains in all seven members of the signal transducer and activator of transcription (STAT) protein family: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Within the transactivation domain, a pivotal tyrosine (Y) residue plays a critical role. Its phosphorylation marks the inception of STAT activation and prompts dimerization between two monomers through a mutual interaction involving the phosopho-Tyr-SH2 domain. In select STAT proteins, a serine (S) residue within the transactivation domain is believed to heighten transcriptional activity upon phosphorylation.

1.5 STAT4 and Interferon type I

Upon the binding of IFN-α/β to IFNAR, a cascade of events is set into motion. This interaction instigates the phosphorylation of STAT1 and STAT4, which activates genes specifically targeted by interferons (34). Remarkably, during the early phases of viral infections, the binding of IFN-α/β and IFNAR predominantly initiate the activation of STAT4, rather than STAT1, particularly evident within natural killer (NK) cells (35). However, IFN-α/β induce IFN-γ production in activated T cells and macrophages, through the IFN-α/β-STAT4 pathway (36, 37) In response to IFN-β, STAT4 takes on a pivotal role within bone marrow-derived mast cells (BMMCs), participants in the immune regulation, encompassing T and B cells, as well as the activation of antigen-presenting cells (APCs) (37). Transitioning into the site of inflammatory cytokines, IL-5 and IL-13, triggering eosinophil activation and enhanced mucus production—defining features of heightened

inflammation. However, a twist unfolds as IFNα/β emerge as the inhibition of IL-5 and IL-13 within peripheral blood mononuclear cells (PBMCs) (38, 39).

The comprehensive examination of target genes associated with SLE through genome-wide profiling has brought into focus the pivotal roles of IRF5 and STAT4 as the most influential susceptibility genes in this condition (40). It is particularly noteworthy that IRF5 operates as a transcription factor within the Toll-like receptor (TLR) signaling pathway, responding specifically to lipopolysaccharide (LPS). Intriguingly, its influence extends beyond direct involvement in the upstream regulation of the IFN-I signaling response. This expansive view is underscored by the identification of more than 7,000 target genes for both IRF5 and STAT4 in PBMCs. These genes, characterized by their involvement in functional pathways associated with the type I interferon system, assume pivotal roles in shaping the inflammatory response observed in SLE, thereby offering deeper insights into the intricate genetic landscape of this autoimmune condition (40).

1.6 STAT4 and dendritic cells

Dendritic cells (DCs) play a pivotal role in initiating and shaping both the innate and adaptive responses. The influential capacities of DCs depend largely on their level of maturation. Mature DCs (mDCs), a proficient subset skilled in antigen-presentation, display distinct characteristics such as an upregulation in proteins related to T-cell activation, including class I major histocompatibility complex (MHC-I) and MHC-II. In comparison to immature dendritic cells (iDCs), mDCs demonstrate substantially higher levels of STAT4 per cell, hinting that the production of STAT4 in DCs is influenced by the degree of maturation (41). This intriguing phenomenon is rooted in the process of DC maturation, where two NF-kB proteins—p65/p50 and p50/p50 dimers—promote the expression of the STAT4 gene by engaging with the transcriptional start site of STAT4 (42). Notably, the presence of Th2 cytokines, namely IL-4 and IL-10, can exert a dampening effect on STAT4 production during DCs maturation (41). Shedding further light, Chiang et al. (43) demonstrated that diminished STAT4 levels correlated with an impaired allo-stimulatory capacity of DCs, suggesting that the STAT4 can substantially affect immunological response of DCs. The balance tips notably as STAT4 predominance over STAT1 in the course of DC maturation, creating a pivotal molecular switch in the network of intracellular signaling pathways. Following activation, mDCs mainly exhibited STAT4-mediated signaling, whereas iDCs lean towards pathways triggered by STAT1 (44)

Inconclusion, STAT4 primarily manifests itself in a variety of immune cell types, with particular emphasis on NK cells, T cells, and B cells. Despite its confirmed presence in human dendritic cells, the functional implications of STAT4 in the context of autoimmune pathogenesis remain shrouded in mystery. Given these gaps in our understanding, we have formulated a hypothesis that revolves around the potential of interferon-β to elevate STAT4 levels within human dendritic cells. This comprehensive study aims to unearth novel insights into the role of STAT4 within autoimmune-mediated dendritic cells, which hold a pivotal position in the IFN-I-mediated inflammation in human immune system.

Chapter 2

Research concept and Methodology

2. Research question

1. Can the IFN-β stimulated purified human dendritic cells enhanced STAT4 expression?
2. What are the roles of STAT4 in the IFN-β stimulated human purified dendritic cells?

3. Research hypothesis

1. Purified human dendritic cells express STAT4 after IFN-β stimulation.
2. STAT4 expressed in IFN-β stimulated human purified dendritic cells promote the IL-12, IL23, and IFN-γ secretion which related to SLE pathogenesis.

4. Research objective

1. To identify the STAT4 expression in IFN-β stimulated human purified dendritic cells
2. To characterize the role of STAT4 expressing in IFN-β stimulated human purified dendritic cells

5. Methodology

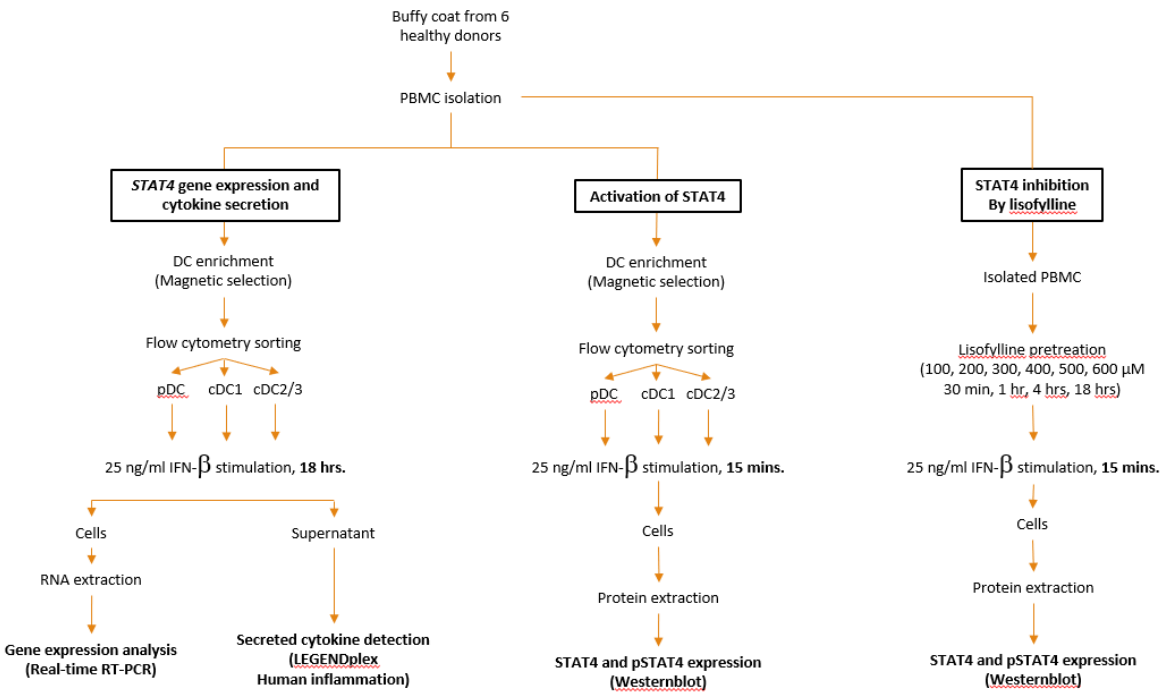


Figure 5 Experimental framework

5.1 Peripheral human DC cells isolation and IFN-α/β stimulation

Buffy coat (n=6) are obtained from healthy blood donors. This study has been conducted under control from the ethical committee at the Blood Center of Thailand (COA No. NBC 2/2021). The PBMCs were isolated using gradient centrifugation and washed two times with s-RPMI (s-RPMI: RPMI supplemented with 10,000 U/mL penicillin and with 10,000 μg/mL streptomycin (Gibco, Thermo-scientific). PBMCs were frozen using 10% DMSO in Fetal bovine serum (FBS) and stored in liquid nitrogen until used.

Total dendritic cells (including pDC, cDC1, and cDC2/3) were enriched by negative selection using EasySep™ Human Pan-DC Pre-Enrichment Kit (Stemcell technologies™), exclude non-DC cells by cocktail antibodies against the markers as shown in table 1, followed by flow cytometry sorting technique. From our preliminary result shows that phosphorylation of STAT4 will occur at 15 min. after IFN-β stimulation, then we decided to stimulate with IFN-β (25 ng/ml.) 15 min. to induce the pSTAT4 protein. For gene expression, we Stimulated with IFN-β (25 ng/ml) 18 hrs. to induce the expression of *STAT4 gene.* Supernatants from cell stimulation were keep at -80 °C for cytokine measurement. In addition, to compare the ability in STAT4 induction, the IFN-α (25 ng/ml) was used to stimulate for 18 hrs. as well

Table 2 Cocktail antibodies against excluded cell markers

Antibodies against	Excluded cells
CD3	T-cell
CD19	MZ B cells, B1 cells, plasma cell
CD14	Monocyte
CD16	Monocyte
CD19	B-cell
CD34	Hematopoietic stem cells
CD56	NK cell
CD66b	Neutrophil
Glycophorin A	Erythrocyte

5.3 Flow cytometry sorting

Total enriched dendritic cells were washed and resuspended with FACs buffer, stained with a set of fluorescent antibodies to sorting out into each dendritic cells sub-population composing anti-CD3 FITC (OKT3; Invitrogen), anti-CD19 FITC (HIB19; Invitrogen), anti-CD56 FITC (TULY56; Invitrogen), anti-HLA-DR APC (L243; Biolegend), anti-CD11c PE (3.9; Invitrogen), anti-CD1c PE-CYN7 (L161; Invitrogen), anti-CLEAC9a APC-EF780 (9A11; Invitrogen), anti-CD123 PE/Dazzle 594 (6H6; Biolegend), anti-CD14 PerCP/Cy5.5 (M5E2; Biolegend), anti-CD16 Alexa flour 700 (B73.1; Biolegend), anti-CD80 Alexa Fluor 488 (2D10; Biolegend), and anti-CD86 APC (IT2.2; biolegend) following gating strategy shown as Figure. 6.

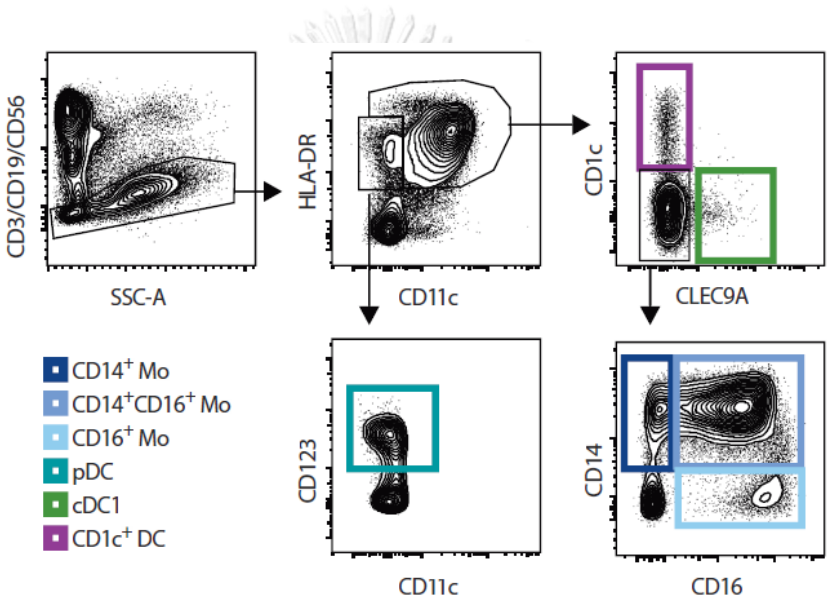


Figure 6 Gating strategy to sorting the DCs into each sub-population (6)

5.4 Western blotting

The stimulated cDC2/3 were lysed by 8 M urea in 100 mM TEAB buffer, sonication by probe to destroy the nucleic acid the condition show in table, Centrifuge the sample with 13,000 g, 4 °C, 10 minutes to drop down the nucleic acid remnant. After that, performing the microBCA (ThermoScientific) for protein quantification. The proteins were stored in -20 °C until use. Loading 30 ug of proteins in each lane and 5 ul of Protein Ladder, 10 to 250 kDa (26619; PageRuler™, Thermo Scientific™). The total protein was separated using SDS-PAGE (130V, 90 mins) and blotted onto nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-rad). The STAT4 and pSTAT4 were detected using rabbit monoclonal anti-STAT4 antibody (C46B10; Cell signaling) and mouse monoclonal anti-pSTAT4 antibody (A19016A; Biolegend). The signal was developed by

secondary antibodies; anti-mouse antibody (800 nm) and anti-rabbit antibody (680 nm). The developed signal was detected and analyzed by Odyssey DLx imaging system (LI-COR) How to measure the intensity of band, please provide more detail.

Table 3 Condition of the sonicator.

Time	30 seconds
Temperature	27 °C
Pulse	On: 10 sec., Off: 5 sec.
Amplitude	35 %

5.5 Semi-quantitative Real-Time RT-PCR

All population of IFN-β stimulated dendritic cells were extracted the RNA using RNeasy kit manufacturer's protocol (Qiagen). The RNA quality is verified by using nanodrop (ThermoScientific™). Acceptable A260/280 are range between 1.4-1.8. The RNA was reverse-transcribed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-rad™). Then qPCR was performed following the SsoAdvanved Universal SYBR Green Supermix (Bio-rad™) with 6 pairs of target gene primer including *GAPDH* was use as the universal internal control to normalize the relative gene expression, STAT*4* was main target of this research, *TNFSF18* was chose as the conventional dendritic cells maturation marker gene (6), *IFN-γ*, the major cytokine of STAT4 activation (45), *IL-12* known as the major mediator of STAT4 activation (46), *IL-23* was Th17 differentiation cytokine which secrete from inflammatory dendritic cells (17).

5.6 Secreted cytokine detection

The cell culture supernatants were thawed completely, centrifuged to remove particulates. Then performed follow the protocol LEGENDplex™ Human Inflammation Panel1 (BioLegend™) by flow-cytometric immunobead assay, which coats the bead with antibody against cytokine composed of IL-1β, IFN-α2, IFN- γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL17A, IL-18, IL-23, IL33.

5.7 Statistical analysis

The semi-quantitative real-time RT-PCR analysis of 6 donors was reported in terms of relative changes in mRNA expression using the 2-ddCT formula (47), followed by an unpaired t-test. The pSTAT4 protein expression was reported in the pSTAT4/STAT4 ratio obtained from the densitometric analyzer. The secreted cytokines were reported using a paired t-test.

Chapter 3

Result

1. The percent estimated of human dendritic cells sub-population isolated from healthy donors

As the estimated dendritic cells subpopulation has not been published yet, we firstly aim to examine the percentage of human peripheral dendritic cells sub-population. According to our two steps purification (magnetic enrichments and flow cytometry sorting), we first check the number of dendritic cells in the positive bead samples to evaluate the efficiency of magnetic beads. The results found that magnetic enrichment allow us to get around 99% of dendritic cells from total 50 million PBMC as recommended in the kit (figure 7A). However, as we would like to obtain the specific sub-population of dendritic cells, we further applied purified dendritic cells to flow cytometry sorting.

By using marker listed in methods section, we obtained dendritic cells (HLA-DR^{+}, CD11c^{+}) around 0.42% in total PBMC. The subpopulation of dendritic cells including plasmacytoid dendritic cells (pDC), conventional dendritic cells (cDC), were 0.07% and 0.35% in total PBMC, respectively. Our results were consistent with previous studies which showed 0.5-1% of dendritic cells, 0.1-0.2% of plasmacytoid dendritic cells, 0.2-0.4% of conventional dendritic cells (Table 4).

Regarding to our knowledge, conventional dendritic cells can be divided into two main sub populations, cDC1 and cDC2/3. We defined them as cDC2/3 because it can separate into 2 subsets by CD5^{+} and CD5^{-}, cDC2 and cDC3 respectively (17), but in our study, we didn't separate out in the limit of cell number. We used CD1c and CLEC9A to differentiate this sub-population. The results found that cDC1 were 0.04% in total PBMC and cDC2 were 0.31% in total PBMC (Figure. 7B-C). These results were calculated from 6 healthy donors.

Table 4 The normal range of the dendritic cells and its subtypes, plasmacytoid DC and conventional DC.

Cells	Normal range in healthy adult	Our result
Dendritic cell (DC)	0.5-1 % (48)	0.42 %
Plasmacytoid DCs	0.1-0.2 % (49)	0.07 %
Conventional DCs	0.2-0.4 % (50)	0.35 %

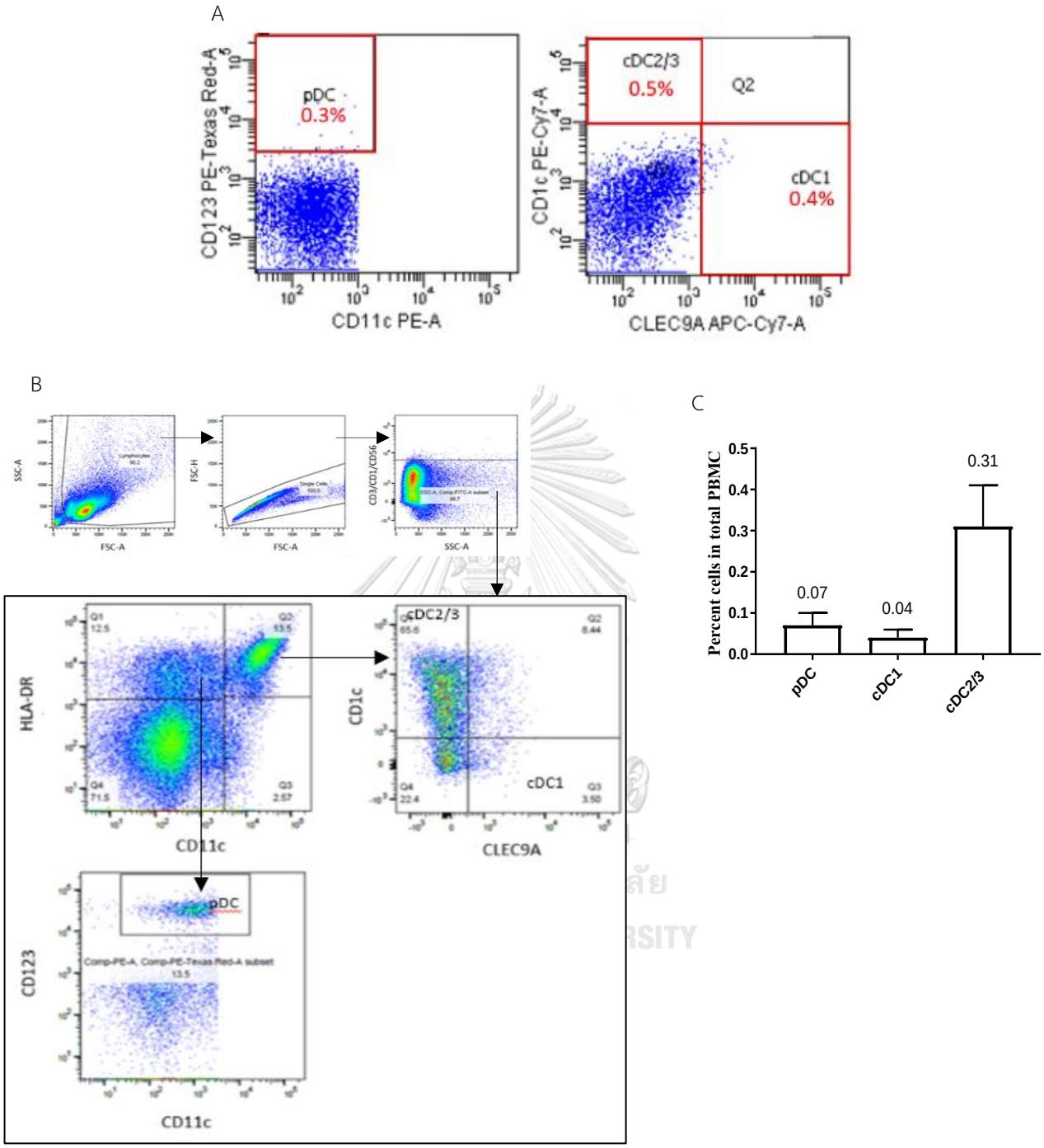
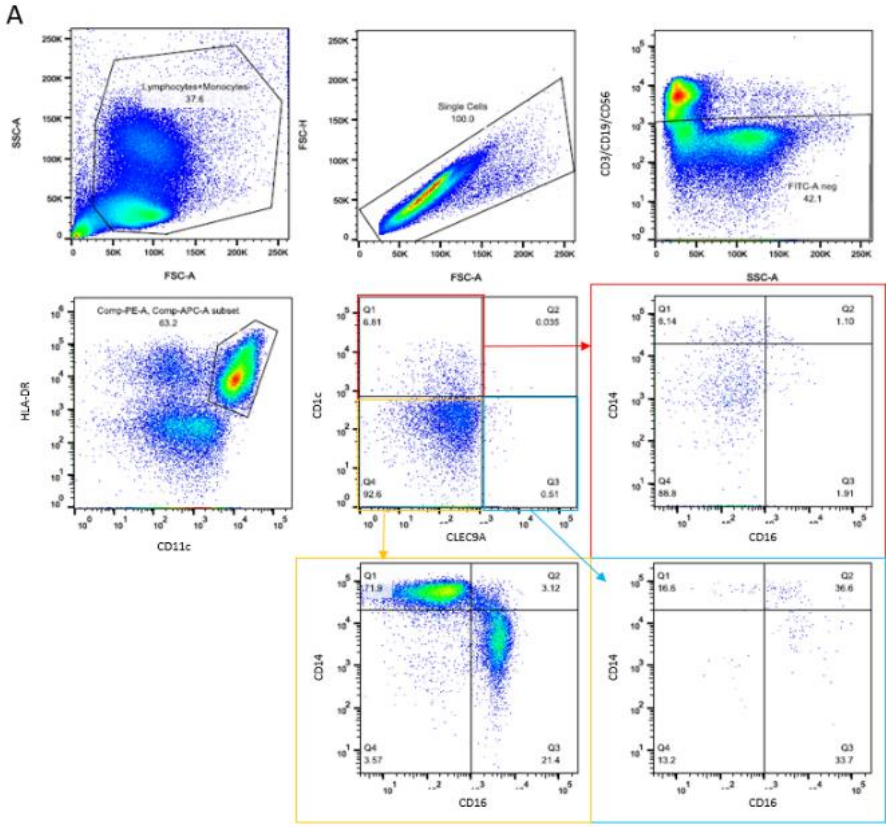


Figure 7 The percent estimated of human dendritic cells sub-population isolated from healthy donors. **(A)** Flow cytometry plot displaying the DC subpopulation in the positive bead sample **(B)** Flow cytometry sorting-based purification of DC subpopulation **(C)** Bar chart illustrating the percentage of dendritic cell subpopulations from the buffy coat of 6 healthy donors using negative selection and flow cytometry sorting. (Due to the absence of markers to differentiate cDC3, this group will be referred to as cDC2/3)

2. The percent of $CD14^+$ and $CD16^+$ presented in dendritic cells sub-population

Since $CD14^+$ and $CD16^+$ were marker commonly used to characterize monocytes and macrophages, we therefore measured the possibility of $CD14^+$ and $CD16^+$ presented in our enriched dendritic cells and isolated dendritic cells fractions. From our enriched dendritic cells, it appears that $CD14^+$ and $CD16^+$ were largely presented in the $HLA\text{-}DR^+$ $CD11c^+$ $CD1c^-$ $CLEC9A^-$ population, which are not classified as dendrite cells. (Figure. 8A, yellow square)

Focusing in our cDC1 fraction, it has expressed CD14 and CD16 2.09% and 44.40%, respectively (Figure. 8B, blue square). While in cDC2/3 fraction, we found CD14 and CD16 were expressed 9.44% and 4.17%, respectively (Figure. 8C, red square). However, it should be noted that, according to a previous report, a study showed that CD14 and CD16 may be expressed on dendritic cells upon induction by an inflammatory microenvironment, especially in patients with SLE with high IFN-β (51). Thus, our analysis may also identify an inflammatory dendritic cell ($CD11c^+$ $CD16^+$ DC). Partially, the healthy dendritic cells could express $CD14^+$ or $CD16^+$ caused by its ontogeny, hematopoiesis process, both macrophage and dendritic cells developed from the same common myeloid progenitor cells (51-54)



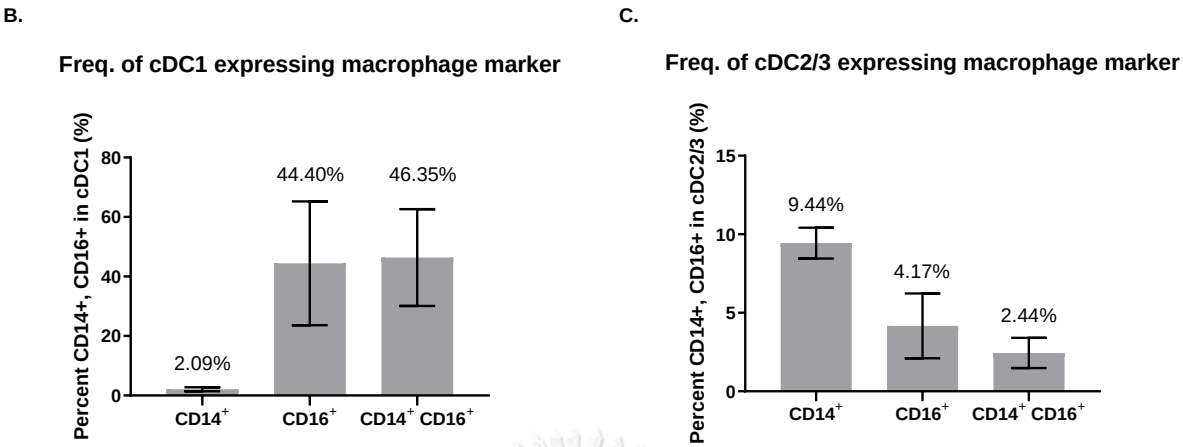


Figure 8 The percent of $CD14^+$ and $CD16^+$ presented in dendritic cells subpopulation.

(A) Gating strategy of CD14 CD16 surface marker among the cDC1 (blue), cDC2/3 (red), Monocyte (yellow) **(B)** Bar chart showing the frequency of CD14 and CD16 surface marker expressing in cDC1 population calculated from 3 donors. **(C)** Bar chart showing the frequency of CD14 and CD16 surface marker expressing in cDC2/3 population calculated from 3 donors.

3. The *STAT4* gene was highly expressed in cDC2/3-treated with either IFN-β or IFN-α

Previous study showed that dendritic cells maturation through inflammatory cytokine and LPS stimulation can trigger *STAT4* expression in human monocyte derived dendritic cells (55). We therefore induced dendritic cells maturation using IFN-β stimulation. According to our preliminary study, PBMC treated with IFN-β can enhanced maturation marker of dendritic cells (HLA-DR and CD86), indicating cell maturation upon IFN-β treatment. (Figure. 9A)

As previous study showed that the dendritic cells stimulated with IFN-β or IFN-α significantly increased *TNFSF18* expression (6). Therefore, *TNFSF18* is used as another indicator to represent the effect of IFN-β stimulation in dendritic cells. As expected, *TNFSF18* expression was significantly higher in conventional dendritic cells subpopulation. Interestingly, we found that *STAT4* was specifically increased only in the cDC2/3 population (Figure. 9B-C*).* This indicated that STAT4 may play some important role in cDC2/3 population maturation.

Regards to previous reports showed that STAT4 induce several inflammatory cytokines, we further investigate the possible down-stream of the activated STAT4 which were composed of *IL-12, IL-23, and IFN-γ*. Unexpectedly, *IL-12*, a major STAT4 inducer, were down-regulated in

IFN-β treated cells. In contrast, *IL-23* and *IFN-γ* were upregulated in cDC2/3 upon IFN-β stimulation (Figure. 9D-F). This indicated that *IL-12* may not be down-stream of STAT4.

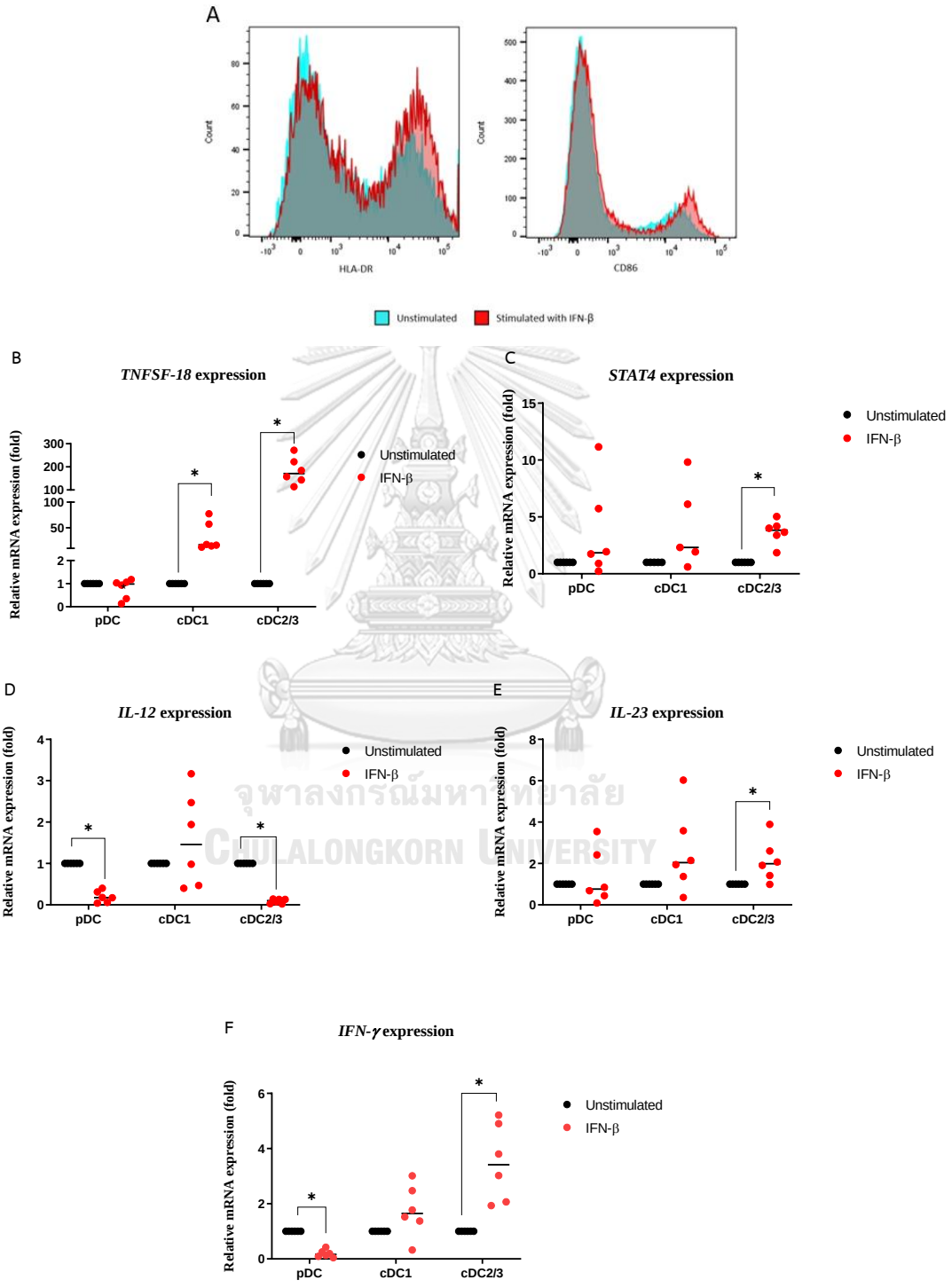


Figure 9 The IFN-β-treated PBMC and gene expression of IFN-β-treated dendritic cells subpopulations. **(A)** Flow histogram showing the expression of HLA-DR and CD86 surface markers in PBMC sample by flow cytometry **(B-F)** The relative mRNA expression of *TNFSF18* **(B)**, *STAT4* **(C)**, *IL-12* **(D)**, *IL-23* **(E)**, *IFN-γ* **(F)** in each dendritic cells subpopulation

(*) statistical significance, p-value < 0.05 by unpair student's t-Test with Prisms software.

4. IFN-β has higher capacity than IFN-α to induce *STAT4* expression.

IFN-α and IFN-β are both type-I IFNs which are capable to induce innate immune response. Moreover, numerous studies have confirmed that IFN-α and IFN-β were elevated in patients with autoimmune diseases and a key mediator in the JAK-STAT signaling pathway (56). In order to compare the effect of both IFN-α and IFN-β on *STAT4* expression, we stimulated cells with IFN-α and IFN-β. *STAT4* genes expression were measured in dendritic cells subpopulations. As expected, both cytokines induced *STAT4* expression significantly in cDC2/3, while IFN-β had more potent effects on *STAT4* induction when compared to IFN-α *(*figure. 10). We therefore mainly used IFN-β in our further experiments.

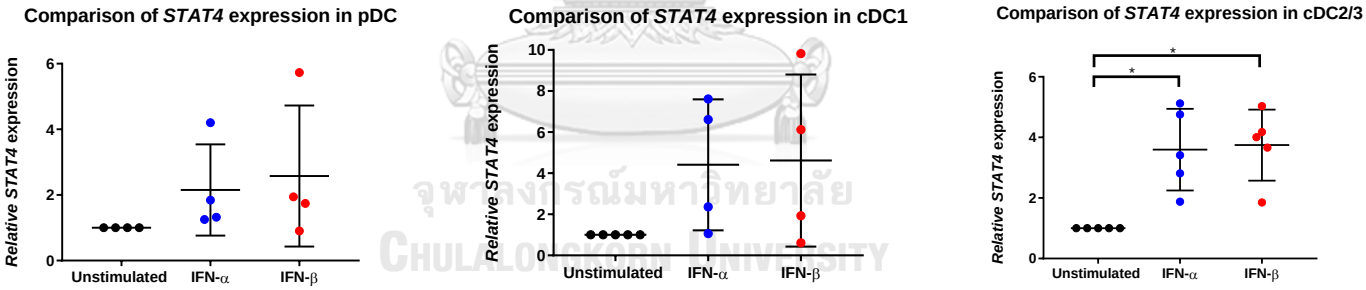


Figure 10 Comparison of ability to induce *STAT4* expression in each subpopulation of dendritic cells. (**Left**: pDC, **Middle**: cDC1, **Right**: cDC2/3) (**Black dot**: unstimulated, **Blue dot**: stimulated by IFN-α, **Red dot**: stimulated by IFN-β)

(*) statistical significance, p-value < 0.05 by unpair student's t-Test with Prisms software.

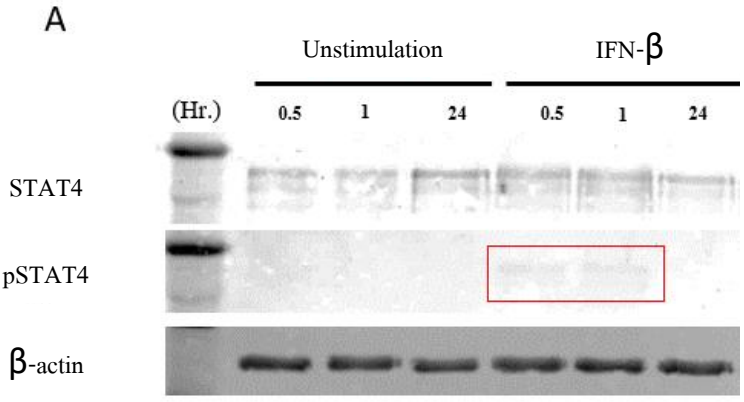
5. The STAT4 transcription factors were phosphorylated after IFN-β stimulation.

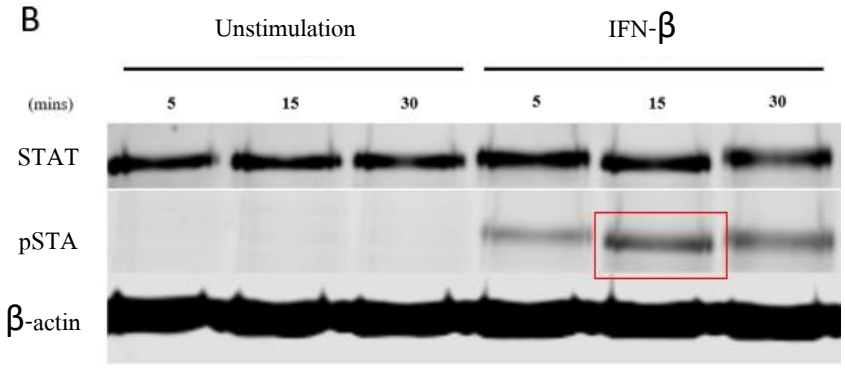
According to our result, *STAT4* were highly upregulated in cDC2/3. We further test whether upregulated STAT4 may conferred to increase the activity of STAT4 transcription factor as well. As protein modification, phosphorylation, is crucial marker for STAT4 activation, we

sorted to detect the STAT4 phosphorylation (pSTAT4) using Western blotting. Initially, we examined the appropriate condition to detect pSTAT4 using PBMC. The PBMCs were stimulated with IFN-β at varying time-points: 30 minutes, 1 hour, and 24 hours. Notably, phosphorylated STAT4 (pSTAT4) exhibited slight expression following IFN-β stimulation at the 30-minute and 1-hour (figure. 11A). To determine a more precise expression interval for pSTAT4, we conducted further investigations at specific time frames – 5 minutes, 15 minutes, and 30 minutes. Interestingly, results demonstrated noticeable pSTAT4 expression upon IFN-β stimulation at the 15-minute interval (figure. 11B). This focused exploration highlights the rapid and time-sensitive nature of pSTAT4 activation in response to IFN-β.

Using the condition found in PBMC, we further investigate pSTAT4 in IFN-β stimulated cDC2/3. We selected only cDC2/3 because the higher expression of *STAT4* in the cDC2/3 group and limitation in the number of purified dendritic cells in another subpopulation.

The results showed that phosphorylated STAT4 were detected in cDC2/3 stimulated with IFN-β *(*figure. 11C-D*)*. This confirmed that IFN-β could enhance the function of STAT4 transcription factor.





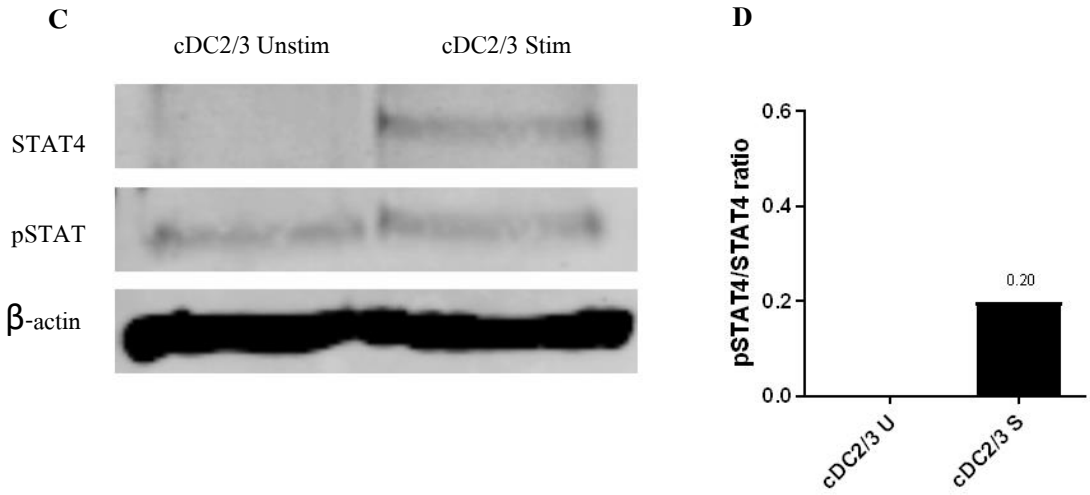


Figure 11 The STAT4 transcription factors were phosphorylated after IFN-β stimulation. **(A)** Western blot results of STAT4 and pSTAT4 protein expression in PBMC upon stimulation with 25 ng/ml. IFN-β for 30 min, 1 h, and 24 h. **(B)** Western blot results of STAT4 and pSTAT4 protein upon stimulation with 25 ng/ml. IFN-β for 5 min, 15 min, and 30 min. **(C)** Western blot results of cDC2/3 stimulation isolated from the blood of three healthy donors. Cells were stimulated with 25 ng/ml IFN-β for 15 minutes, 81 kDa pSTAT4 and 81 KDa STAT4 protein compared with beta-actin as an internal control. **(D)** Densitometry of protein intensity by Li-Cor Odyssey CLx and calculated as pSTAT4/STAT4 ratio showed that pSTAT4 was higher in activated cDC2/3.

6. Lisofylline could not completely inhibit STAT4 activation via IFN-β stimulation in human PBMCs

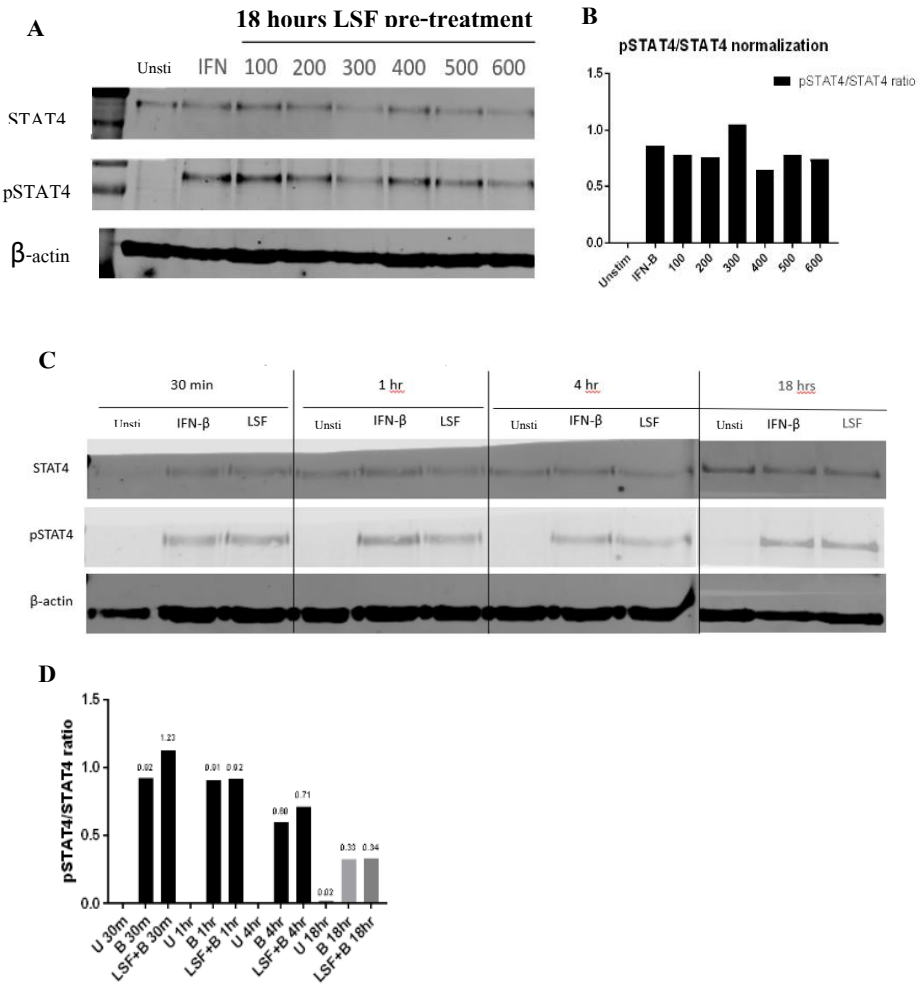
To investigate the function of STAT4 in human cDC2/3, a targeted inhibition of STAT4 was carried out utilizing a chemical reagent known as lisofylline. Previous studies have established that a concentration of 100 μM lisofylline specifically inhibits the pSTAT4 protein in mouse splenocytes (57). This approach was employed to elucidate the precise role of STAT4 in human cDC2/3 and provide insights into its potential regulatory functions (57).

We firstly test the suitable concentration of Lisofylline for inhibiting STAT4 expression in PBMC, we initially conducted a pre-treatment phase with varying concentrations of LSF on IFN-β treated PBMCs. Concentrations of 100, 200, 300, 400, 500, and 600 μM were administered, with an 18-hour interval preceding IFN-β stimulation. Surprisingly, our findings yielded unexpected

results, as the administered LSF did not effectively inhibit the phosphorylation of STAT4 (figure. 12A-B).

Next, we proceeded to elucidate the optimal timing for lisofylline (LSF) to inhibit STAT4 activation in PBMC. Employing a highest concentration of 600 μM LSF, we conducted pre-treatment on PBMCs for varying durations: 30 minutes, 1 hour, 4 hours, and 18 hours. This pre-treatment was carried out prior to stimulation with 25 ng/ml IFN-β for a duration of 15 minutes (figure. 12C-D). Analyzing the experimental outcomes, we observed no discernible differences in STAT4 activation between the pre-treated and untreated LSF groups at any of the designated time points. Lastly, we try to confirm that LSF not able to block pSTAT4 in our condition, we pretreat the PBMCs with 150 μM LSF for 1 hr. then stimulate with 25 ng/ml IFN-β or 2 ng/ml IL-12 as positive control for 15 min. the result show that pSTAT4 did not decrease after LSF pretreatment. Moreover, pSTAT4 was not activated after stimulate with IL-12 that we use as the positive control, this conflict might cause by the quality of the cytokine.

Based on the findings of these results, the investigators arrived at the conclusion that lisofylline was ineffective in repressing STAT4 expression in human PBMCs. Consequently, it could not serve as a viable tool for studying the role of STAT4.



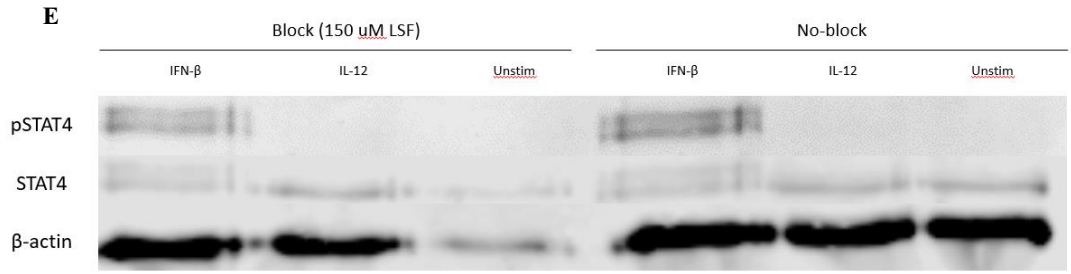


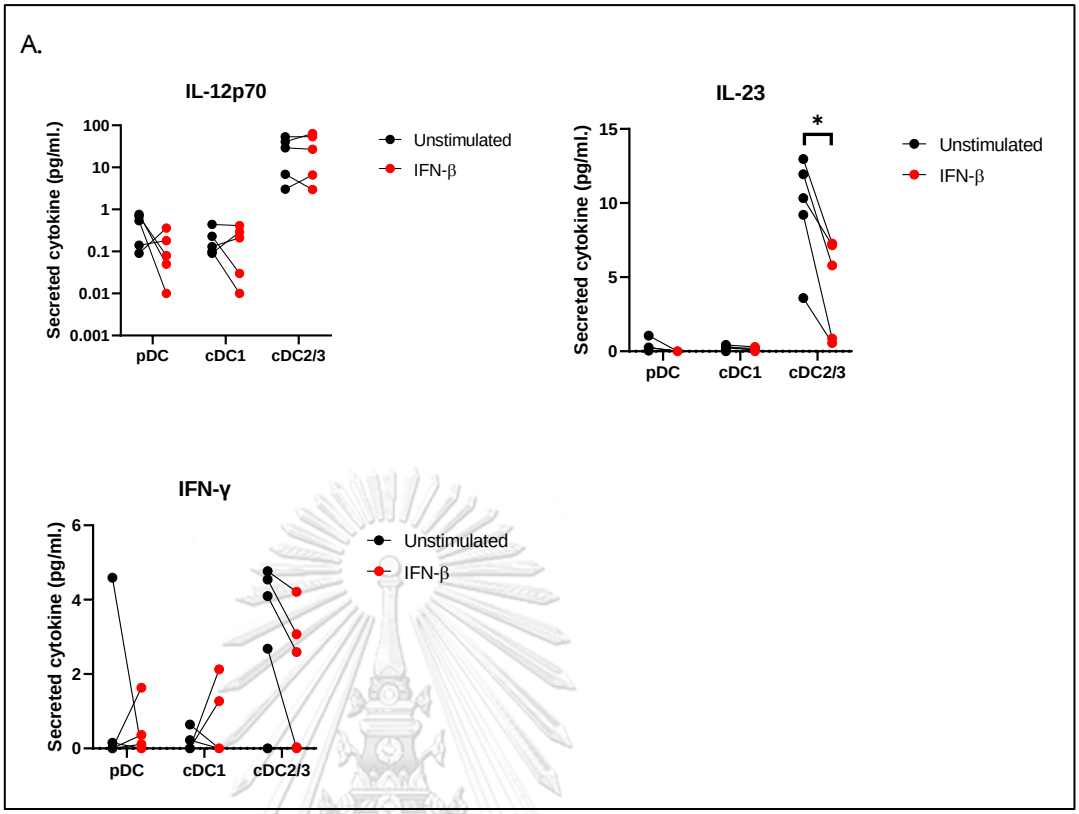
Figure 12 The result of lisofylline pretreatment

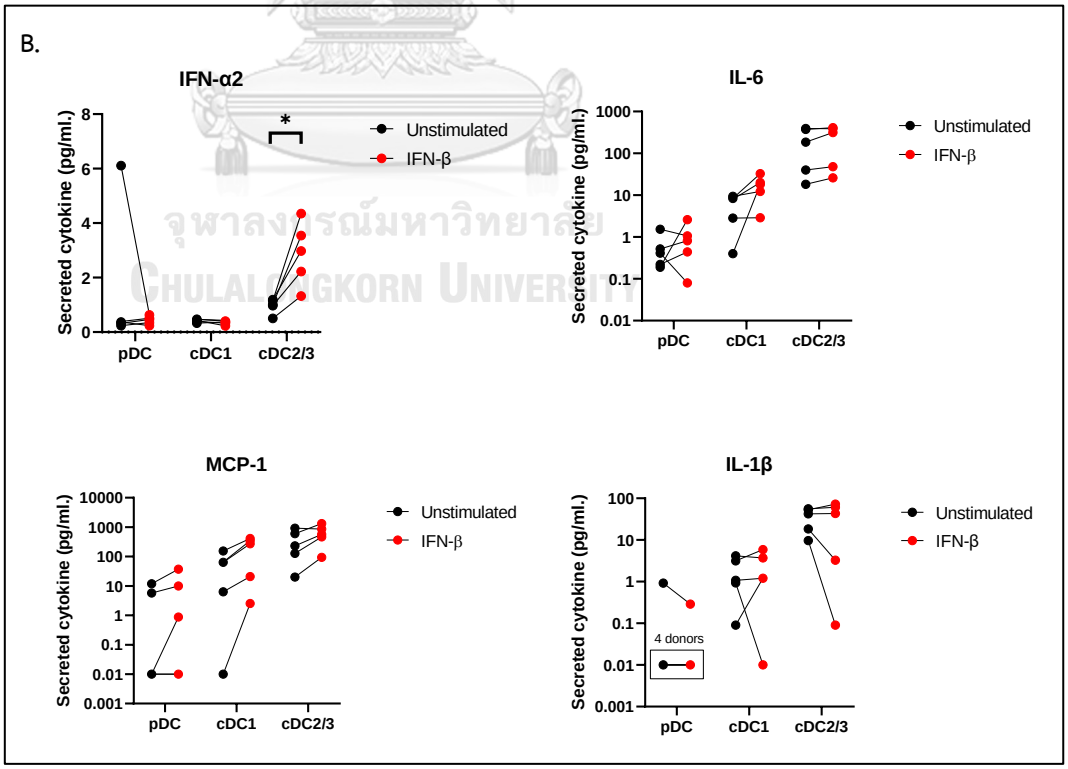
(A) Western blot results from cell culture in LSF at various concentrations. **(B)** Chart showing the pSTAT4/STAT4 ratio shown in Fig. 12A. **(C)** Western blot results from cell culture at *600* μM LSF at 30 min, 1 h, 4 h and 18 h. **(D)** Chart showing the pSTAT4/STAT4 ratio shown in Fig. 12C. **(E)** Western blot result from cell culture in IFN-β and IL-12 by block and no-block with LSF (U: Unstimulation, B: stimulated with IFN-β without LSF pretreation, LSF: pre-treated with LSF before IFN-β stimulation)

7. The IFN-α was secreted in IFN-β stimulated human cDC2/3 but not pDC and cDC1.

By elucidating the association of STAT4 activation and cytokine release, we collected culture supernatant from the subpopulation DC treated IFN-β and profile of cytokine IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL17A, IL-18, IL-23, IL33 were determined using LEGENDplex™ human inflammation panel 1.

Unexpectedly, we found that IFN-β stimulation resulted in a noteworthy reduction in the amount of secreted IL-23 (*p*-value = 0.006) (figure. 13A). With the same direction, TFN-α, IL-10, IL-17A, IL18, were also experienced a decrease (figure. 13C). Among those cytokines, we noticed that *IL-23* expression was clearly contrast to the levels of their corresponding cytokine. This might occur as the sorted cDC2/3 express CD14 slightly, the inflammatory marker defined by Dutertre et al. They demonstrated that $CD14^+$ cDC2/3 can promote Th17 differentiation by IL-23, but not $CD14^-$ cDC2/3 (17). On the contrary, cDC2/3 upon exposure to IFN-β stimulation showed increasing in the IFN-α2 release, (*p*-value = 0.01) (figure. 13B). These results may indicate that, in terms of autoimmune development, IFN-β-stimulated cDC2/3 might operate in an inflammatory signal expansion manner.





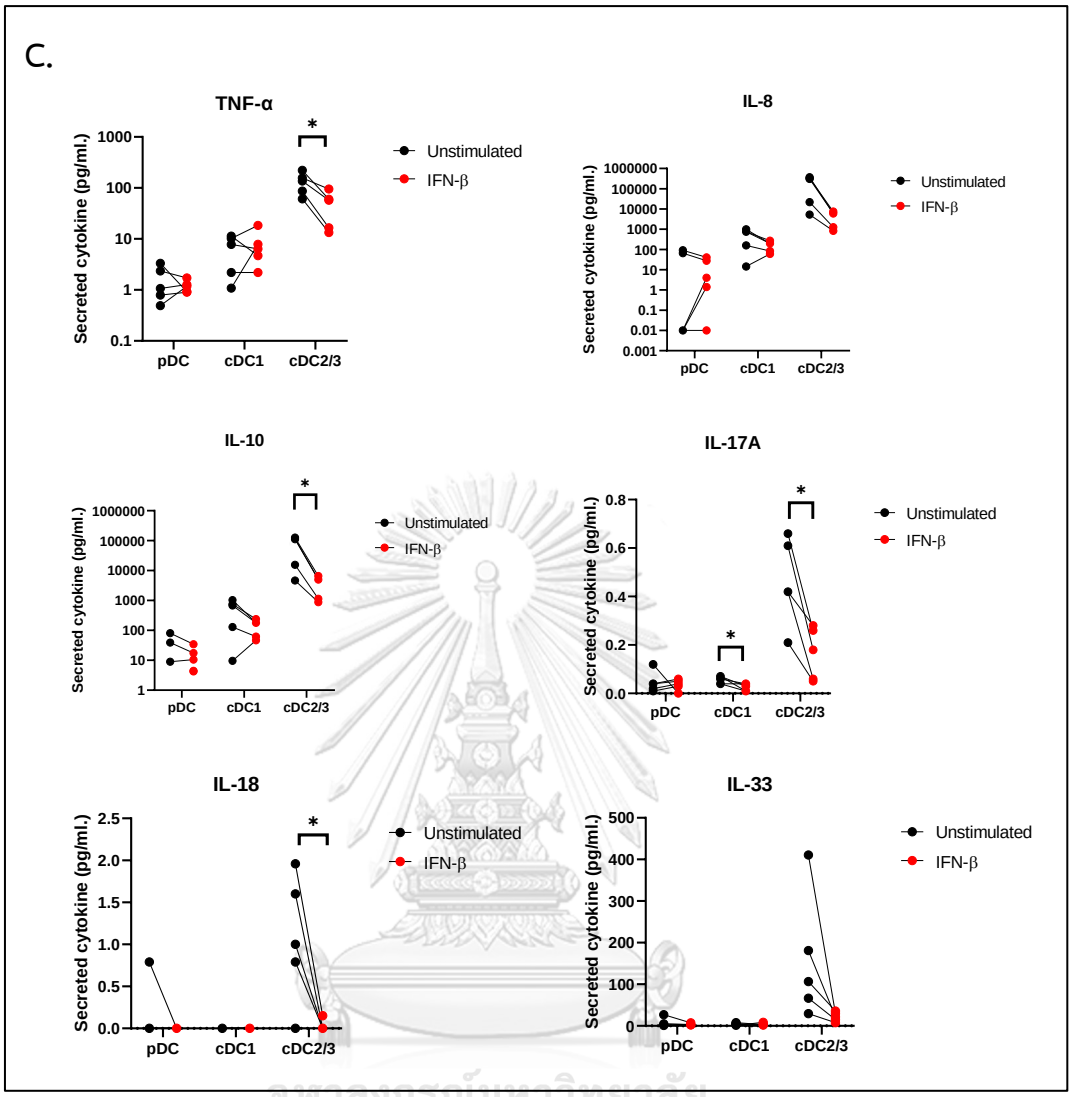


Figure 13 The dot plot illustrates the analysis of cytokine secretion in dendritic cell subpopulations from 5 donors

(A) Depicts cytokines based on target genes expression analysis. **(B)** Represents a cluster of cytokines that experience elevation following IFN-β stimulation. **(C)** Depicts a cluster of cytokines that decrease after IFN-β stimulation

Chapter 4

Discussion and Conclusion

In our research, we have successfully purified a specific subpopulation of dendritic cells to a quantity that closely resembles the normal range found in healthy individuals. It's worth noting that there isn't yet a widely published exact count for the cDC2/3 subpopulation, as it is a relatively newly explored area of study. Most research has focused on investigating its functions and its relevance to various diseases. This lack of published data has led investors to adopt an approach of combining the counts of cDC1 and cDC2/3 as a way to estimate the proportion of conventional dendritic cells. This strategy provides a practical workaround for assessing the presence of these cell types despite the current gap in precise numerical information. However, it should be noted that our data were obtained from only 6 healthy donors. Further study to increase number of sample size may need to provide the accurate dendritic cells population number.

A distinct subpopulation of dendritic cells (DCs) in human PBMC, which express the CD11c and CD16 marker but not CD1c called "$CD16^+$ DC". These $CD16^+$ DCs exhibit heightened levels of HLA-DR, CD86, and CD40, this seems like a subpopulation of cDC1 ($CD11c^+$ $CD1c^-$ population) compared to our result (figure 7B). They are noteworthy for their strong ability to stimulate T cells. Additionally, they are known to generate significant amounts of inflammatory cytokines like TNF-alpha, IL-6, and IL-12 when exposed to TLR agonists (58-60), thereby exhibiting a pro-inflammatory role. Thus, this type of DC can be detected in healthy blood donors.

However, a crucial point to consider is that $CD16^+$ $CD11c^+$ cells might not be true DCs but could be a subgroup of $CD16^+$ non-classical monocytes. The $CD16^+$ DC subpopulation identified by Villani et al. (51) displays notable differences in their response to IFN-I signaling, viral stimuli, and gene expression related to the migration of immune cells compared to $CD16^+$ non-classical monocytes, despite sharing certain transcriptional profile similarities. Notably, based on the expression of specific surface markers such as CD16, CD11b, CD14, and CD36, $CD16^+$ DC could also potentially describe as a subpopulation of $CD16^+$ monocyte which called $Slan^+$ cells. These $Slan^+$ cells were initially thought to be a type of CD16-expressing DC, but they are now associated with monocyte identity (52-54) In essence, this subpopulation of dendritic cells that identified by their CD16 expression, presents an intriguing area of study. Their unique characteristics, intricate relationships with monocytes, and potential overlap with other cell types

like Slan$^+$ cells underscore the complexity of immune cell subpopulations and warrant further investigation. Our study revealed an interesting insight regarding the high expression of CD16$^+$ within the cDC1 subpopulation. Contrary to initial assumptions of a flow cytometry analysis error at all, our findings suggest that this elevated CD16$^+$ expression might actually be attributed to the presence of a distinct CD16$^+$ DC subpopulation that had been previously identified.

Distinct from the singular cDC1 population, peripheral blood cDC2s can be further divided into two subsets: cDC2-A and cDC2-B (also known as DC2 and DC3, respectively, in a study by Villani et al. (51)). These subsets, both characterized by CD1c expression, exhibit varying genetic and functional traits. Specifically, cDC2 (CD32b$^+$) stands out with higher levels of CD11c, CD1c, and MHC class II genes. On the other hand, cDC3 (CD36$^+$ CD163$^+$) showed elevated expression of inflammatory genes, closely resembling the expression profile of CD14$^+$ classical monocytes (51)**.** This differentiation into cDC2 and cDC3 aligns with earlier observations of cDC2s being categorized into CD5^{hi} and CD5^{lo} groups, cDC2 and cDC3 respectively. Additionally, these subsets correspond to putative CD14$^+$ and CD163$^+$ divisions within CD1c-expressing cells (cDC3) (61, 62)**.** Moreover, a comprehensive analysis of human blood DCs using mass cytometry by Hernandez et al. also unveils distinct populations of cDC2s, defined by CD163 and signal regulatory protein a (SIRPa) expressions (63). In this context, CD11c$^+$ CD163$^-$ and CD11c$^+$ CD163$^+$ loosely mirror the characteristics of cDC2/3. This adaptability is understandable, given the notable variability in the cDC2 population among different individuals (63)**.** In summary, the elevated expression of the CD14$^+$ marker observed within the cDC2/3 population is a typical phenomenon. This is because CD14$^+$ can also serve as a surface marker for a type of cell called monocyte, specifically referred to as CD14$^+$ monocyte-like DC. However, it's important to note that the presence of genuine monocytes has been effectively distinguished and excluded through the utilization of CD1c$^+$.

Prior investigations have provided valuable insights into the role of STAT4 expression within dendritic cells that are stimulated by IL-12 and originate from monocytes. These studies have uncovered a captivating pattern: the activation of STAT4 was exclusively observed in dendritic cells that had matured. This activation process was contingent upon the maturity status of the cells, which necessitated the activation of antigens through molecules referred to as PAMPs, stemming from microbial infections. Upon encountering these antigenic triggers, a series of events unfolded, leading to the release of pro-inflammatory cytokines such as IFN-I, IL-23, IL-2, IL-17, and IL-35. These cytokines have undergone extensive examination and have been

demonstrated to possess the ability to induce STAT4 activation, thus reinforcing their crucial role in the regulation of immune responses (29, 41) Furthermore, in the context of human myeloid cells, encompassing peripheral blood monocytes and monocyte-derived dendritic cells, a distinct behavior emerged when exposed to IL-12 under normal circumstances. In the absence of maturation stimuli such as exposure to LPS (lipopolysaccharide) or IFN-γ, IL-12 failed to initiate the phosphorylation of STAT4 in this specific lineage of cells. Interestingly, a contrasting observation emerged as these cells attained a state of maturity: phosphorylated STAT4 manifested in response to the dual stimulation of IL-12 and IFN-α, revealing an intricate interplay between cytokine stimulation and cellular maturation (64). Shifting our focus to human lymphoid cells, previous investigations have illuminated the behavior of resting T cells in response to IL-12. It was discovered that resting T cells exhibited minimal expression of IL-12R at detectable levels. However, upon activation of the T cell receptor through the use of IL-2, a notable transformation occurred: the expression of IL-12R reached a level where it became responsive to IL-12 stimulation (65, 66) All the evidence suggests that both human lymphoid and myeloid cells need to mature and activate, respectively, before they can trigger the IL-12-induced STAT4 signaling pathway.

This research is a direct exploration of *STAT4* expression obtained from 6 healthy donors. The researchers discovered that when cDC2/3 were exposed to IFN-β, there was a noticeable increase in the expression of *STAT4*, which was not observed in other groups of dendritic cells. This intriguing distinction might be attributed to IFN-β ability to induce maturation, although perhaps not as strongly as previously observed with LPS or IFN-γ in earlier studies (64). Corresponding to previous study (67), from the Figure 4A of our result, showing the activation marker of dendritic cells, CD86, displaying a slight increase after IFN-β stimulation. This finding raises the possibility that the relatively modest increase in CD86 might contribute to the absence of phosphorylated STAT4 expression in most cell groups, except for cDC2/3 (6). Consequently, this points towards the idea that IFN-β is particularly effective in prompting a mature state specifically in cDC2/3, leading to a significantly heightened expression of the *STAT4* expression (67), as evident in Figure 9C. The significance of these observations lies in the immunomodulatory potential exhibited by cDC2/3 through the STAT4 signaling pathway. This pathway's influence extends beyond merely triggering autoimmune diseases that can harm the host. Given that STAT4 plays a pivotal role in governing the production of IFN-γ, it also stimulates the proliferation of Th1 cells, which could contribute to the disease activity observed in patients.

In the part of the role of STAT4 investigation. To inhibit STAT4 expression, we utilized a chemical inhibitor called lisofylline (LSF). A previous investigation conducted on IL-12-stimulated mouse splenocytes revealed a decrease in STAT4 expression as the concentration of LSF increased. This suggests that LSF's impact on splenocytes is influenced by its dosage (57). In a separate human study, it was demonstrated that LSF can diminish the secretion of IFN-γ in IL-12-treated effector T-cells (68) This implies that LSF might impede the STAT4-induced IFN-γ secretion, given that STAT4 is recognized as the primary signal transducer for IL-12. Furthermore, another study exhibited that treating human IL-12-treated B cells with LSF resulted in reduced levels of pSTAT4, without affecting overall mRNA and protein levels (69). However, our experimental findings contrast with these prior studies. In human PBMC, there was no reduction in STAT4 and pSTAT4 expression as LSF concentration increased. The researchers postulated that LSF's inhibitory mechanism does not directly target STAT4 protein expression or the *STAT4* gene. Instead, LSF was employed to hinder STAT4 protein expression triggered by IL-12. Notably, this distinction lies in our study, which used IFN-β stimulation. Nonetheless, the outcome of our study, indicating that LSF does not inhibit STAT4 expression under IFN-β stimulation, presents an intriguing challenge for future investigations into STAT4 inhibitors within the IFN-I stimulation pathway.

In the population of cDC2/3 cells, the decrease in IL-23 secretion may be due to the fact that 90% of the purified cDC2/3 cells belong to the non-inflammatory type ($CD14^-$ cDC2/3), as defined by Dutertre et al (17). This non-inflammatory type is distinct from the inflammatory type ($CD14^+$ cDC2/3), which tends to find in inflammatory microenvironments such as those observed in SLE patients (17). In an experiment where $CD4^+$ naïve T-cells were co-cultured with $CD14^+$ cDC2/3 cells, it was demonstrated that $CD14^+$ cDC2/3 cells have the potential to promote the differentiation of Th17 cells, typically through the action of IL-23. This was evident from the high levels of IL-17 detected in the co-culture supernatant. In contrast, when $CD14^-$ cDC2/3 cells were co-cultured, a significantly lower level of IL-17 was detected (17). In summary, the non-inflammatory cDC2/3 cells may secrete IL-23 at a lower level than the inflammatory cDC2/3 cells. Following stimulation with IFN-β, there was a notable increase in the secretion of IFN-α. Usually, during a viral infection, IFN-α is the primary type of interferon almost secreted by pDCs in response. However, it's important to note that not only pDCs, but also cDCs, have been observed in previous studies to secrete IFN-α, albeit in smaller quantities (70). Moreover, in situations where other cell types were infected with the virus, IFN-β was also identified as a

cytokine that prompted cDCs to secrete IFN-α. This finding aligns with the results from our experiments.

In conclusion, this investigation provides evidence of STAT4 expression in cDC2/3 upon IFN-β stimulation, as effectively as IFN-α. STAT4 was activated in cDC2/3 upon IFN-β treatment, not dependent on IL-12. This led to cDC2/3 producing more IFN-α but not IFN-γ. This gap could be filled by further research, investigating pSTAT4 binding sites on DNA to identify genes directly expressed from pSTAT4. Additionally, we demonstrated that LSF cannot inhibit STAT4 activation induced by IFN-β in PBMC. Our results contribute another step towards a better understanding of STAT4 in dendritic cells in terms of autoimmune disease development.

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