Identification of a new disease gene for a familial autoimmune disease

Miss Thivaratana Sinthuwiwat

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biomedical Sciences

Inter-Department of Biomedical Sciences

GRADUATE SCHOOL

Chulalongkorn University

Academic Year 2020

การค้นหายีนใหม่ของโรคภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเองชนิดที่ถ่ายทอดทางกรรมพันธุ์

น.ส.ทิวารัตน์ สินธุวิวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ สหสาขาวิชาชีวเวชศาสตร์
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2563

Thesis Title	Identification of a new disease gene for a familial autoimmune disease
By	Miss Thivaratana Sinthuwiwat
Field of Study	Biomedical Sciences
Thesis Advisor	Professor VORASUK SHOTELERSUK, M.D.
Thesis Co Advisor	Professor KANYA SUPHAPEETIPORN, M.D. Ph.D.

Accepted by the GRADUATE SCHOOL, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the GRADUATE SCHOOL

(Associate Professor THUMNOON NHUJAK, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman

(Professor APIWAT MUTIRANGURA, M.D. Ph.D.)

..... Thesis Advisor

(Professor VORASUK SHOTELERSUK, M.D.)

..... Thesis Co-Advisor

(Professor KANYA SUPHAPEETIPORN, M.D. Ph.D.)

..... Examiner

(Professor NATTIYA HIRANKARN, M.D. Ph.D.)

..... Examiner

(PRASIT PHOWTHONGKUM, M.D.)

..... External Examiner

(Associate Professor Wallaya Jongjaroenprasert, M.D.)

ทิวารัตน์ สินธุวิวัฒน์ : การค้นหายีนใหม่ของโรคภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเองชนิดที่ถ่ายทอดทางกรรมพันธุ์. (Identification of a new disease gene for a familial autoimmune disease) อ.ที่ปรึกษาหลัก : ศ. นพ.วรศักดิ์ โชติเลอศักดิ์, อ.ที่ปรึกษาร่วม : ศ.ดร. พญ.กัญญา ศุภปีติพร

โรคภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเอง ถูกกระตุ้นได้โดยปัจจัยทางพันธุกรรมและสิ่งแวดล้อม ส่งผลให้เกิดความล้มเหลวของระบบภูมิคุ้มกันซึ่งพัฒนาให้เกิดภาวะตอบสนองต่อแอนติเจนของตนเอง โดยแสดงลักษณะตอบสนองภูมิคุ้มกันต่อเนื้อเยื่อของตนเอง จนถึงปัจจุบันสาเหตุของการเกิดโรคยังไม่เข้าใจแน่ชัด คณะผู้วิจัยได้ศึกษาครอบครัวใน 3 รุ่นที่มีสมาชิกจำนวน 9 คนได้รับความเจ็บปวดจากหนึ่งในสามของโรคภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเอง ประกอบด้วย กลุ่มโรคต่อมไทรอยด์อักเสบจากภูมิคุ้มกัน โรคต่อมไทรอยด์เป็นพิษ หรือ โรคเอสแอลอี โดยทำการค้นหา genetic variant และศึกษากลไกที่อาจเกี่ยวข้องกับการเกิดโรคภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเองในครอบครัวใหญ่นี้ คณะวิจัยได้ศึกษาลักษณะ immunophenotype ของเซลล์ภูมิคุ้มกันของผู้ป่วยด้วยเทคนิค flow cytometry และใช้วิธี Whole-genome linkage (WGL) และ whole-exome sequencing (WES) ในการค้นหา genetic variant ในผู้ป่วย 9 คนในครอบครัวนี้ รวมถึงศึกษากระบวนการฟอสโฟรีเลชันของโปรตีนที่เกิดการกลายพันธุ์และ downstream protein ที่เกี่ยวข้องอีกด้วย อีกทั้งยังใช้ single-cell RNA sequencing ศึกษา peripheral blood mononuclear cell จากผู้ป่วย 3 คน และ control โดยผลการศึกษา Immunophenotype ไม่พบความแตกต่างทั้งในด้านจำนวนและการแสดงออกของยีน *ILT2* ในเซลล์ผู้ป่วยของผู้ป่วยเมื่อเทียบกับ control ผลการวิเคราะห์ WGL แสดงตำแหน่งที่เกี่ยวข้องบนโครโมโซม 19 โดยมีค่า LOD score สูงสุดเท่ากับ 2.04 และผลของ WES พบการกลายพันธุ์แบบเฮเทอโรไซกัส c.479G>A (p. G160E) ในยีน *ILT2* อยู่บนตำแหน่งที่เกี่ยวข้องโดยพบในผู้ป่วยที่เป็นโรคภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเองทั้งหมด และยืนยันการกลายพันธุ์ที่พบโดยวิธี PCR-Sanger sequencing โดยการกลายพันธุ์ segregate กับลักษณะอาการของโรคในครอบครัวด้วยสัดส่วนการแสดงออก 75% penetrance ผลการศึกษาการทำงานของโปรตีนในเซลล์ jurkat ที่ใส่ลักษณะกลายพันธุ์เข้าไปเปรียบเทียบกับที่ใส่ลักษณะปกติ พบว่ามีการลดลงของฟอสโฟรีเลชั่นทั้งโปรตีน *ILT2* และ *SHP-1* ซึ่งเป็น downstream molecule ของ *ILT2* นอกจากนี้ผลของ scRNA-seq พบว่าเซลล์กลุ่ม M2 monocyte มีจำนวนเพิ่มขึ้นในผู้ป่วย 3 คนเมื่อเทียบกับ control เป็นที่น่าสังเกตว่าการศึกษาก่อนหน้านี้แสดงให้เห็นว่ามีการเพิ่มขึ้นของเซลล์กลุ่ม M1 monocyte ซึ่งมีบทบาทสำคัญกับภาวะการอักเสบในโรคภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเองในผู้ป่วยเมื่อเทียบกับ control การศึกษานี้สรุปได้ว่า การกลายพันธุ์ของยีน *ILT2* ส่งผลให้เกิดการลดลงของฟอสโฟรีเลชั่นในโปรตีน *ILT2* และ *SHP-1* และอาจเกี่ยวข้องกับโรคภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเองในครอบครัวนี้ การศึกษานี้เป็นครั้งแรกที่แสดงให้เห็นถึงความเกี่ยวข้องว่า ยีน *ILT2* มีความเป็นไปได้ที่จะเป็นยีนใหม่ที่ก่อให้เกิดภูมิคุ้มกันต่อต้านเนื้อเยื่อตนเอง

สาขาวิชา	ชีวเวชศาสตร์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5887776920 : MAJOR BIOMEDICAL SCIENCES

KEYWORD: Autoimmune diseases ILT2 sc-RNA sequencing

Thivaratana Sinthuwiwat : Identification of a new disease gene for a familial autoimmune disease. Advisor: Prof. VORASUK SHOTELERSUK, M.D. Co-advisor: Prof. KANYA SUPHAPEETIPORN, M.D. Ph.D.

Background: Autoimmune diseases, triggered by genetic and environmental factors resulted in the failure of the immune system to develop tolerance toward self-antigens, are characterized by immune responses against self-tissues. Until now the cause of autoimmune disease is not well understood. Here, we identified a three-generation family with 9 members suffering from one of the three autoimmune diseases – Grave's disease, Hashimoto thyroiditis, or SLE. Objective: To identify a genetic variant underlying the autoimmune diseases of this large family and to determine its molecular pathomechanism. Methods: Immunophenotype including flow cytometry was characterized. Whole-genome linkage study (WGL) and whole-exome sequencing (WES) of all 9 affected family members were performed. Phosphorylation of the mutated and its downstream proteins were studied. Single-cell RNA sequencing (scRNA-seq) was studied using peripheral blood mononuclear cell of three patients and a healthy control. Results: Immunophenotype shows no differences in percentage and expression of *ILT2* in immune cells. WGL revealed a locus on chromosome 19 with the maximum LOD score of 2.04. WES identified a heterozygous missense mutation, c.479G>A (p. G160E) in *ILT2*, located within the linked region, in all affected members. PCR-Sanger sequencing confirmed the identified mutation. The mutation segregated with the disease phenotype in family with 75% penetrance. Jurkat cells transfected with the mutant *ILT2*, compared to those with wild-type *ILT2*, showed decreased phosphorylation of both *ILT2* and *SHP-1*, an ILT2's downstream molecule. scRNA-seq showed significantly increased percentage of M2 monocyte subsets in the three patients, compared to the control. Notably, M1 monocyte subsets, which was previously observed to play a role in inflammation and autoimmune diseases, in patients were not different from the control. Conclusion: A variant in *ILT2* leading to decreased phosphorylated *ILT2*, and *SHP-1* is associated with autoimmune diseases in a large family. This is the first time to implicate ILT2 as a possible new disease gene for autoimmunity.

Field of Study:	Biomedical Sciences	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to thank HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy for giving me the opportunity to being a student here and providing the funding for the study. A very special gratitude goes out to all down at by the Thailand Research Fund and the Chulalongkorn Academic Advancement into Its 2nd Century Project for providing the funding for the work. I would like to express my sincere gratitude and appreciation to my thesis advisor, Prof. Vorasuk Shotelersuk for his invaluable advice. I am most grateful for his teaching and endless inspiration without his supervision, it would not be impossible to complete my thesis. I am very thankful to Prof.Kanya Suphapeetiporn for her advice and as well as giving me a good positive mindset in life and at work. In addition, I am grateful to Dr. Supranee Buranapraditkun for invaluable helping me in the part of Flow cytometry analysis. It was fantastic to have the opportunity to work majority of my research in her laboratory facilities.

I would also like to thank the member of Excellence Center for Medical Genetics, King Chulalongkorn Memorial Hospital including Dr. Rungnapa Ittiwut, Dr. Chupong Ittiwut, Dr. Chureerat Phokaew, Mr. Chalurmpon Srichomthong, Miss Adjima Assawapitaksakul, Miss Wanna Chetruengchai, Miss. Kanyanut Thaweerachatham, and others person who helps me by providing their assistance and time, especially to Dr. Siraprapha Tongkobpetch for always listening and assisting as well as giving me the valuable suggestion. I am also grateful to Miss Netchanok leela-adisorn and Dr. Praewphan Ingrungruanglert the staffs of Stem cell and cell therapy research center, Faculty of Medicine, Chulalongkorn University for assisting and providing time to my work.

I would also like to thank my friends, Dr. Wandee Udomchaiprasertkul and Miss Phanasit Poowasanpetch for encouragement and for pushing me further than I thought I could go. Finally, I most gratefully acknowledge my family for all their support throughout the period of this research. I would not have achieved this far, and this thesis would not have been completed without all the support that I have always received from them.

Thivaratana Sinthuwiwat

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Background and rational

Autoimmune diseases result from the failure of the immune system to develop tolerance toward self-antigens. They are characterized by the activity of autoreactive lymphocytes, which cause tissue or organ damage through the generation of antibodies that react against host tissues, or effector T cells, which are specific for endogenous self-peptides. Genetic and environmental factors cooperate in the induction of autoimmunity(1). Examples of autoimmune diseases include rheumatoid arthritis, systemic lupus erythematosus (SLE), Hashimoto's thyroiditis (HT), and Graves' disease (GD).

Hashimoto's thyroiditis (HT) and Graves' disease (GD) is two major subtypes of autoimmune thyroid diseases (AITD), which have the generation of cellular or humoral immune responses against the thyroid gland in common. Hashimoto's thyroiditis (HT) is inflammation of the thyroid gland, which can lead to hypothyroidism. On the other hand, Graves' disease is an autoimmune disease that leads to a generalized overactivity of the entire thyroid gland, thus developing hyperthyroidism (2). Systemic lupus erythematosus (SLE) is an autoimmune disease that mainly affects the joints and organs, including skin, kidneys, brain, blood vessels, and serous membranes. It has been widely proposed that the development of this condition results in the complex interaction between genetic factors and individual susceptibility to environmental

factors. SLE is an autoimmune disorder characterized by the presence of autoantibodies directed against nuclear antigens (3).

Until now, the cause of the autoimmune disease is not known. However, our group has collected a family with an autoimmune disease whose inheritance showing an autosomal dominant mode. Diagnosis of autoimmune disease was confirmed based on a review of medical records and interviews with patients by the physician. Interestingly, the first study identifies a new gene that can contribute to the immunopathogenesis of familial autoimmune disease, including autoimmune thyroid disease and SLE.

We aimed to identify the new gene of familial autoimmune disease using whole-exome sequencing (WES) combined with whole-genome linkage analysis. Whole-Genome Linkage Analysis, is a well-established statistical method for mapping heritable trait genes to their chromosome locations, was used to find critical chromosome regions. Whole-genome linkage analysis in this family revealed one linkage loci on chromosome 19 with a maximum value of linkage with the LOD score of 2.04.

Whole exome sequencing (WES), which provides a potential disease-causing mutation, was used to investigate the variants. For these candidate genes with possible pathogenic mutations, prediction programs and a search for the variants in controls were performed. As a result, we identified c.479G>A variant in the immunoglobulin-like transcript receptor 2 (*ILT2*) or leukocyte Ig-like receptor 1 (*LILRB1/CD85j*) in this family. It changes a nucleotide at position 479 from G to A (c.479G>A), leading to amino acid changing from glycine to glutamic acid at

position 160 (p.G160E). The mutation is located on the immunoglobulin-like domain (Ig-like C2-type 2), an important region for binding with a major histocompatibility complex, class I.

Immunoglobulin-like transcript receptor 2 (*ILT2 /LILRB1/CD85j*) is an inhibitory receptor broadly expressed on leucocytes and recognizes human antigen G (HLA-G). *ILT2* is expressed on various proportions of NK cells, CD8+ lymphocytes, and CD4+ lymphocytes, as well as B lymphocytes, monocytes, and dendritic cells. In this study, the percentage of each immune cell and *ILT2* expression in peripheral blood mononuclear cells (PBMC) of autoimmune patients were analyzed by flow cytometry (4).

Single-cell RNA sequencing (scRNA-seq) has begun to define the phenotypes of distinct immune cell populations in PBMC of several autoimmune diseases (5). Furthermore, scRNA-seq provides an unbiased approach to define, characterize disease-related immune cell subset and identify the transcriptional signatures of these immune cells that may be correlated with pathogenicity (6).

Although the regulatory role of *ILT2* may contribute to the immunopathogenesis of autoimmune thyroid disease and SLE has been identified (4, 7), its pathomechanism is still unknown. Based on the previous report, the molecular mechanisms after activation of *ILT2* showed that a crosstalk between *ILT2* and TCR leads to the phosphorylation status of *ILT2*, which recruits *SHP-1* and results in reduced phosphorylation of TCRz and linker for activation of T cells. *ILT2* ligation inhibited both signaling and cellular events important for T cell activation (8).

Our research group has identified the candidate genes *ILT2* for autoimmune disease. We also demonstrate functional effects of this variant in familial autoimmune disease.

Keyword

Autoimmune disease, *ILT2,* single cell RNA sequencing

Abbreviation

Hashimoto's thyroiditis: HT , Graves' disease: GD, Systemic lupus erythematosus: SLE, Immunoglobulin-like transcript receptor 2: *ILT2*

Research questions

1. Is the c.479G>A variant in *ILT2* identified in a large family with autoimmune disease, pathogenic?

2. What is the possible pathomechanism of this variant for causing familial autoimmune disease?

Hypothesis

The c.479G>A variant of ILT2 leads to a defective ILT2 receptor on lymphocyte, which diminishes regulatory functions of the immune cell leading to overactivity of the immune.

We hypothesize that loss-of-function mutations of ILT2 in the immunopathogenesis of the familial autoimmune disease.

Objective

To determine the pathogenic role and the effects of the identified c.479G>A variant in ILT2 in patients with familial autoimmune disease.

Research design

Descriptive and *in vitro* studies

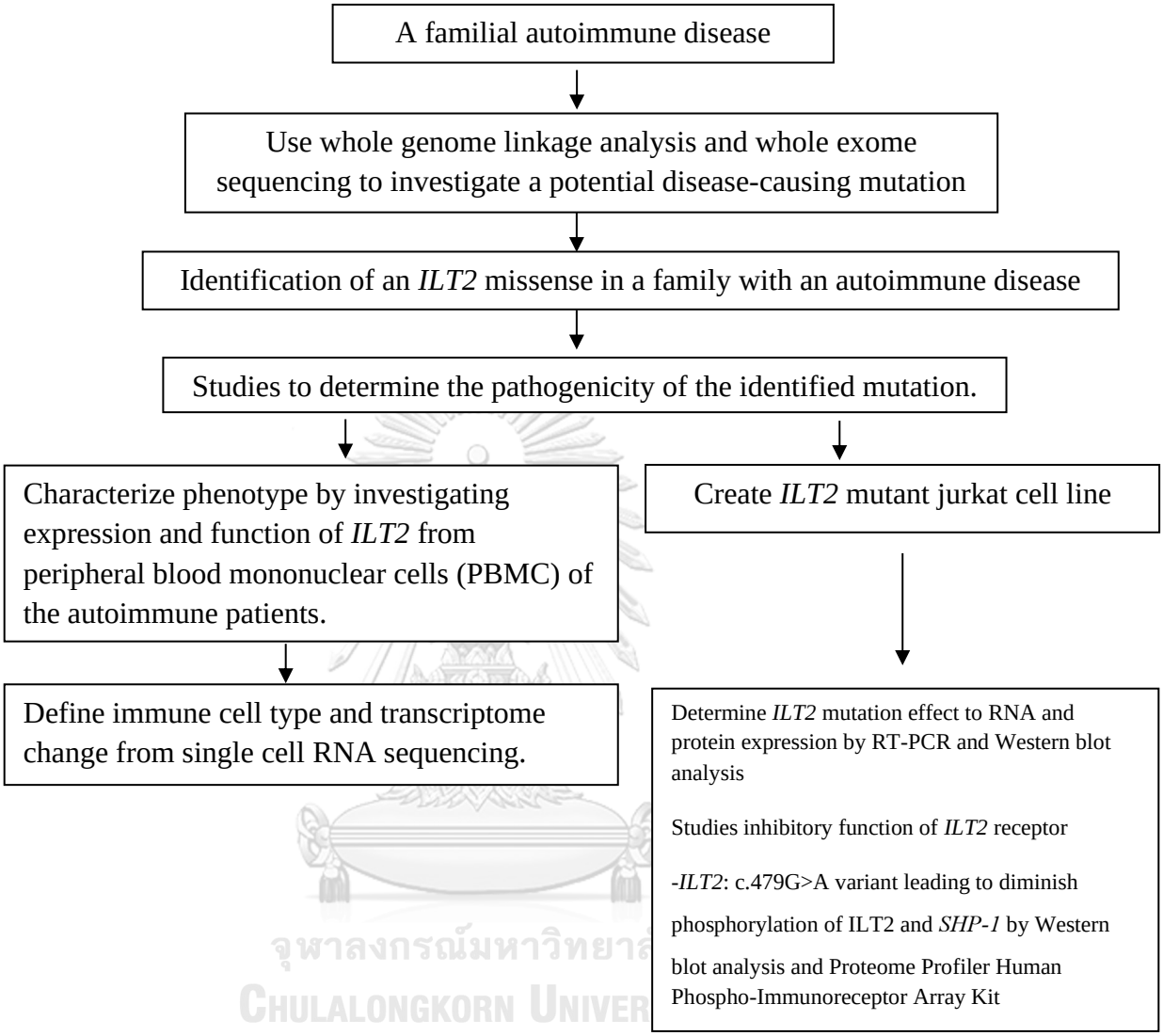
Ethical consideration

This study has been approved by the local Ethics Committee. Written informed consent was obtained from patient who participated in the study.

Expected benefit and application.

This research has a potential to identify a new human disease gene for an autoimmune disease. It may provide evidence that patients with the autoimmune disease have an abnormal function of *ILT2*, possible pathogenesis of the autoimmunity.

Conceptual Framework



CHAPTER II

REVIEW LITERATURE

Autoimmune disease

Autoimmune diseases result from the failure of the immune system to tolerance self-antigens and characterized by the activity of autoreactive lymphocytes, which cause tissue or organ damage through the generation of antibodies that react against host tissues, or effector T cells, which are specific for endogenous self-peptides. Genetic and environmental factors cooperate in the induction of autoimmunity (1). Autoimmune diseases are classified as organ-specific or systemic autoimmunity. Organ-specific autoimmunity involves chronic T-cell or antibody targeting of a particular organ. By contrast, systemic autoimmunity is often the result of the breakdown of immunological tolerance to ubiquitous self-molecules, which generates autoantigen- mediated damage in multiple organs at the same time (2).

Autoimmune thyroid diseases

Hashimoto's thyroiditis (HT) and Graves' disease (GD) are the most common organ-specific autoimmune disease and the two major subtypes of the autoimmune thyroid diseases (AITD), which generate of cellular or humoral immune responses against the thyroid gland. AITD is relatively common, affecting up to 5% of the general population, and more frequent in women by a ratio of 9:1 (9). HT is characterized by massive immune cell infiltration of the thyroid gland. Activated CD8+ and CD4+ T cells, B cells, plasma cells and macrophages infiltrate to thyroid glands leading to thyrocyte destruction and contribute inflammation leading to

hypothyroidism. Most HT patients have autoantibodies to thyroglobulin (Tg) and thyroperoxidase (TPO) and have been proposed to participate hypothyroidism by blocking thyroid-stimulating hormone (TSH) receptor (2, 9). On the other hand, the main immunological feature of GD is the presence of autoantibodies directed against the TSH receptor, which stimulate the growth and function of thyroid cells, thus leading to overactivity of the entire thyroid gland thus resulting in the development of hyperthyroidism. The autoimmune process that conducts to GD is believed, to begin with, the activation of thyroid-specific CD4+ T cells, followed by recruitment into the thyroid of autoreactive B cells and production of anti-thyroid antibodies (10) (Figure 1).

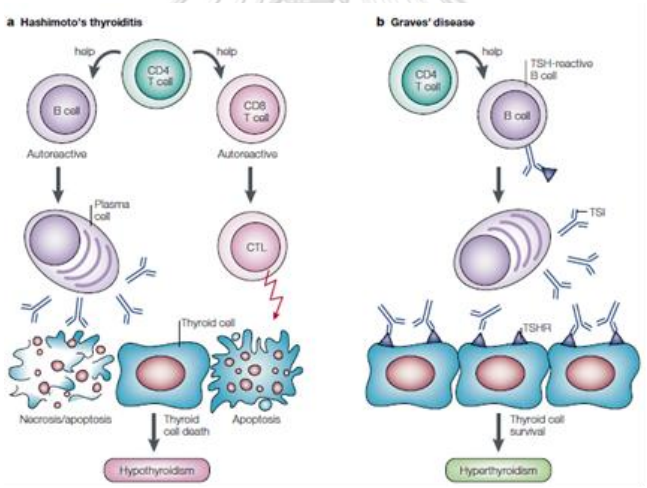


Figure 1. Thyroid autoimmunity produces two opposite pathogenetic processes and clinical outcomes.

Although the etiology of these conditions has not been elucidated, different factors seem to contribute to their pathogenesis, including genetic factors, environmental factors, stress, and cytokines. Genetics plays a crucial role in the pathogenesis of AITD. Many immune-related genes have been implicated in genetic susceptibility to AITD. These genes include polymorphisms of

CTLA4 and CD40 genes, the presence of HLA-B8, HLA-DR3, HLA-DR4, and HLA-DR5 alleles. (11-13)

Systemic lupus erythematosus (SLE)

SLE is an autoimmune disease that affects multiple organs, including joints, skin, kidneys, brain, blood vessels, and serous membranes (3). It has been widely proposed that the development of this condition results in the complex interaction between genetic factors and individual susceptibility to environmental factors. The main immunological alterations of SLE are characterized by autoantibodies directed against nuclear antigens, polyclonal activation of B lymphocytes, and the impaired function of T cells (7). The prevalence of SLE varies with ethnicity but is estimated to be about 1 per 1000 overall, with a female-male ratio of approximately 10:1 (14). Nowadays, more than 30 genetic variant associations with SLE have been identified since genome-wide association studies became possible in 2007. Most SLE-associated gene products participate in key pathogenic pathways, including Toll-like receptor and type I interferon signaling pathways and immune regulation pathways such as *HLA* and *Fc*γ receptor genes, *IRF5*, *STAT4, PTPN22, TNFAIP3, BLK, BANK1, TNFSF4 and ITGAM* (15)(Figure 2).

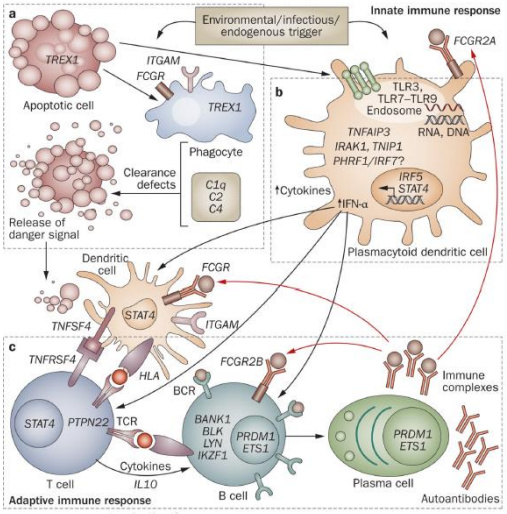


Figure 2. Model of SLE-associated genetic variants in the immune response.

Immunoglobulin-like transcript receptor 2 (ILT2 /LILRB1/CD85j) and autoimmune disease

A critical phenomenon in peripheral tolerance is inhibition of T cell activation, which is of great importance in the pathogenesis of autoimmune diseases (16). Other molecules are also able to suppress the activation of T cells, including the leukocyte Ig-like receptors (LIRs) or immunoglobulin-like transcript receptors (ILTs) (Figure 3) (17).

ILT2 is an inhibitory receptor widely expressed on leukocytes and binds to different class I HLA molecules, including human leukocyte antigen G (HLA-G) (18, 19). ILT2 encoded within the leukocyte receptor complex (LRC) on 19q13.4. ILT2 is a membrane molecule of 110 kDa with an extracellular region of 442 amino acid, which includes four immunoglobulin-like domains, a 25- amino acid hydrophobic transmembrane domain, and a 167-amino acid cytoplasmic domain (17, 20).

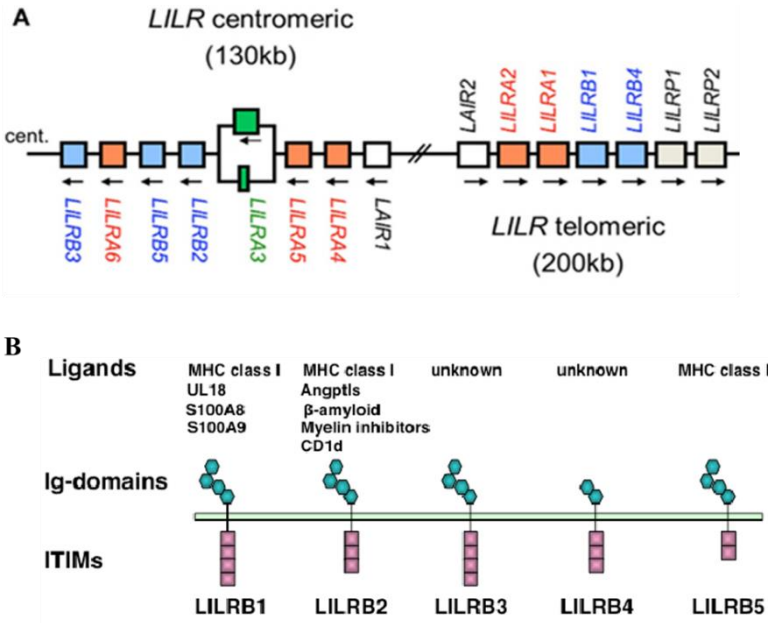


Figure 3 . Schematic representation of the LRC on human chromosome 19q13.4

(A) A large number of Ig-like receptor genes, including two clusters of LILR loci are encoded within the LRC (20). (B) Schematic of the ligands, extracellular Ig-domains (hexagons), and intracellular ITIMs (boxes) of human LILRBs. (21)

ILT2 receptor is detected on monocytes, macrophages, dendritic cells (DCs), and B cells. Furthermore, this receptor is expressed by a subset of T lymphocytes, approximately 4–20%, and approximately 75% in NK cells (7). *ILT2* binds to different class I HLA molecules, including HLA-G.

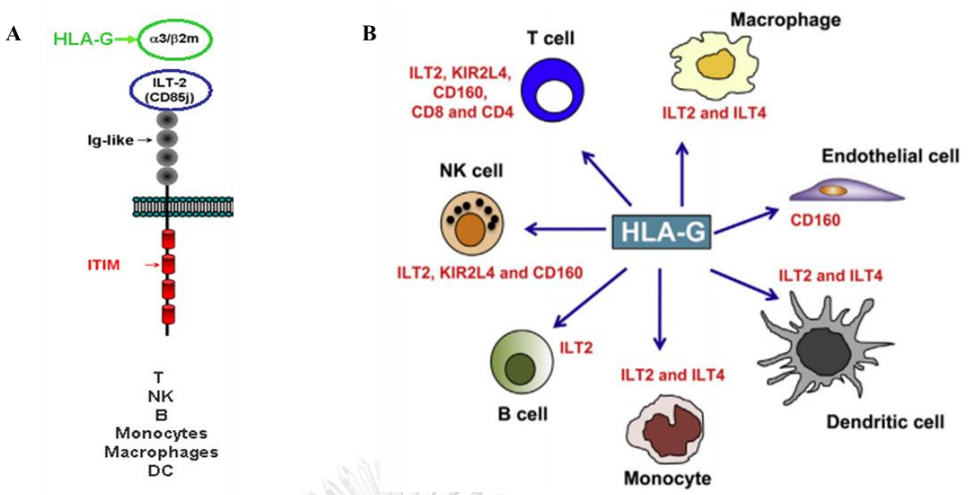


Figure 4. ILT-2 and its ligand.

ILT2 receptors were shown on the cell surface of T cells, NK cells, B cells, monocytes, dendritic cells, and macrophages (19).

Its intracytoplasmic domain contains two pairs of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that, upon phosphorylation, recruits the Src homology containing protein-tyrosine phosphatase SHP1 and SHP2, which involves in the inhibition of different intracellular signal pathway (22). Moreover, *the ILT2* receptor exerts a negative regulatory effect on the activation of T cells by inhibiting the phosphorylation of the TCR ζ-chain, the recruitment of Zeta-chain-associated protein kinase 70 (ZAP-70), and the phosphorylation of the linker for activation of T cells (LAT) and extracellular signal-regulated protein kinase (ERK1/2) (8).

Furthermore, it has also been demonstrated that *ILT2* inhibits cytotoxicity and chemotaxis of NK cells to their target cells. In addition, *ILT2* has a tolerogenic effect in DCs by inhibiting the production of IL-12. Moreover, in B cells, this receptor can down-regulate their

activation and proliferation as well as the intracellular signals generated through the B cell receptor (BCR) (Figure 5) (23). Finally, *ILT2* on monocyte/macrophage induces macrophage differentiation toward an M2 phenotype and enhances macrophage IL-6 and CXCL1 secretion (24).

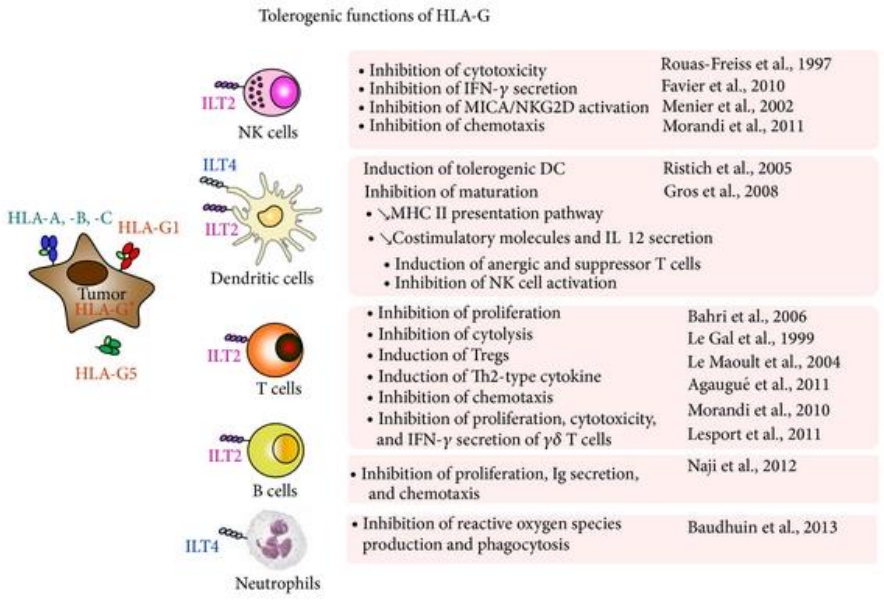


Figure 5. *ILT2*/HLA-G interaction.

HLA-G-mediated immunosuppressive effects through engagement of inhibitory receptors *ILT2* expressed by immune cells such as T cells, NK cells, B cells, macrophages and neutrophils.

Doniz-Padilla et al. (4) analyzed the expression of *ILT2* in various cell populations from the peripheral blood of autoimmune thyroid disease (AITD) patients and healthy subjects. Flow cytometry analysis exhibited significantly higher expression of *ILT2* in the T lymphocyte subsets (CD4+and CD8+) as well as in NK cells (CD56+) from Hashimoto's disease (HT) and Graves' disease (GD) patients compared with controls ($P<0.05$ in all cases).On the contrary, the levels of

expression of *ILT2* in B cells (CD19+) and monocytes (CD14+) were not different in the three groups studied. However, they found the significantly diminished inhibitory activity of *ILT2* on cell proliferation in autoimmune thyroid disease patients. In addition, they found a diminished regulatory function of *ILT2* on the synthesis of cytokines such as IL10, which mainly exert an anti-inflammatory role in AITD patients. In summary, they provide evidence that the abnormal expression and function of *ILT2* detected in AITD suggests that this receptor could participate in the pathogenesis of thyroid autoimmunity.

In this regard, Monsiváis-Urenda A et al. have previously described a decreased expression of this regulatory receptor and significantly diminish the inhibitory activity of *ILT2* in the CD4+and CD8+ from patients with systemic lupus erythematosus (SLE) (7). In 2013, Monsiváis-Urenda A et al. also evaluated the expression and function of *ILT2* in dendritic cells and found significantly higher levels of circulating plasmacytoid DCs (pDCs) in SLE patients. Conversely, the expression of *ILT2* was diminished in both pDCs and myeloid DCs (mDCs) from SLE patients. Their results suggest that *ILT2* participates in the defective immune regulation observed in patients with SLE (25).

Regulatory T cells (Tregs) are immunosuppressive cells that play a crucial role in regulating immune tolerance in pathological settings and preventing autoimmune diseases. However, their frequency and functional capacities in various autoimmune immune diseases are still controversial, and their exact roles and molecular mechanisms have not been fully defined (26) . In addition, HLA-G binds to *ILT2* on NK cells, T cells, and macrophages, inhibits the

cytotoxicity of NK cells and CD8+ T cells, and causes an increase in the percentage of Treg cells in the population, and thereby contributes to immune tolerance (27).

Marazuela et al. (28) found different regulatory T cell subsets present in the peripheral blood and thyroid tissue from patients with autoimmune thyroid disease. Some of these cell subsets are abundant and able to synthesize TGF-beta. However, the suppressive function of these cells seems to be defective. However, less information is available regarding these *ILT2*+ Treg cells in autoimmune disease.

The previous study indicates that LAP is an authentic marker able to identify a TGF beta-expressing CD4+CD25+Treg subset. Moreover, Glycoprotein A repetitions predominant (GARP) is a transmembrane protein containing leucine-rich repeats. Recently, the surface of activated Tregs is highly express GARP. Higher purity Treg can isolate with the combination of GARP and LAP+. Likewise, GARP is a cell surface molecule of Tregs that maintains its regulatory function and homeostasis. GARP and LAP are well-characterized late-stage Treg activation markers, and they contribute directly to a contact-dependent TGF-β-mediated suppressive mechanism in Tregs (29).

Bossowski et al. showed a significant decrease of CD19+ CD24hiCD27+ IL-10+ and CD19 + IL-10+ B lymphocytes in untreated patients with GD and HT in comparison to the healthy controls. They concluded that the reduced number of Breg cells with expression of CD19+ CD24hiCD27+ IL-10+ and CD19+ IL10+ (B10) could be responsible for loses immune tolerance and development of the autoimmune process in thyroid disorders (30).

The role of *ILT2* in the inhibition of the different lymphocyte subsets was studied by using the HP-F1 is an anti-*ILT2*, a monoclonal antibody (mAb) that mimics the ligand and inhibits the proliferation induced through CD3/TCR (7) in peripheral blood mononuclear cells from patients with systemic lupus erythematosus (SLE).

Dietrich et al. found Upon tyrosine phosphorylation of *ILT2*, ITIMs recruit phosphatases Src homology protein (SHP)-1 and/or -2, which can dephosphorylate molecules involved in immuno-tyrosine activation motif (ITAM)-induced signaling pathways and inhibits TCR Signaling by reduces phosphorylation of TCRζ, LAT, and ERK1/2. In addition, using Pervadanate, they showed that tyrosine phosphorylation was required for *SHP-1* recruitment to *ILT2* (8).

Single cell RNA sequencing (scRNA-seq)

In recent years, scRNA-seq has significantly advanced our knowledge of biological systems. New technology allows for the investigation of heterogeneity in cell populations, identify and characterizes disease-related immune cell subsets. Furthermore, this technique can explore pathological populations contributing to disease and the transcriptional signatures of immune cells correlated with pathogenicity. scRNA-seq has begun to define the phenotypes of distinct immune cell populations in PBMC of several autoimmune diseases (5). scRNA-seq is making further essential discoveries of new lymphocyte subpopulations and states, their molecular underpinnings, and their relationship to physiology and disease (31). Based on the

transcriptional signatures of such immune cells, the data reveal inflammation and novel biomarkers (32).

Arazi et al. (33) analyzed kidney samples and peripheral blood cells from patients with lupus nephritis and control subjects using single-cell RNA sequencing. They identified 21 subsets of leukocytes in lupus nephritis, including multiple populations of myeloid cells, T cells, natural killer cells, and B cells that demonstrated both pro-inflammatory responses and inflammation-resolving responses. In addition, almost all immune cell subsets in the kidney showed elevation of type I interferon.

CHAPTER III

MATERIALS AND METHODS

1. Participants

A total of 9 autoimmune disease patients are composed of 7 are autoimmune thyroid disease (AITD) patients. In addition, 3 with Hashimoto's thyroiditis (HT), 4 with Graves' disease (GD), and 2 are systemic lupus erythematosus (SLE) that fulfilled the diagnostic criteria for AITD and SLE were studied. Peripheral blood samples were obtained from all individuals, including history. In all cases, written informed consent was obtained; their blood samples were collected, and genomic DNA and RNA were extracted using Extraction Kits (Qiagen Inc., Valencia, Calif., USA).

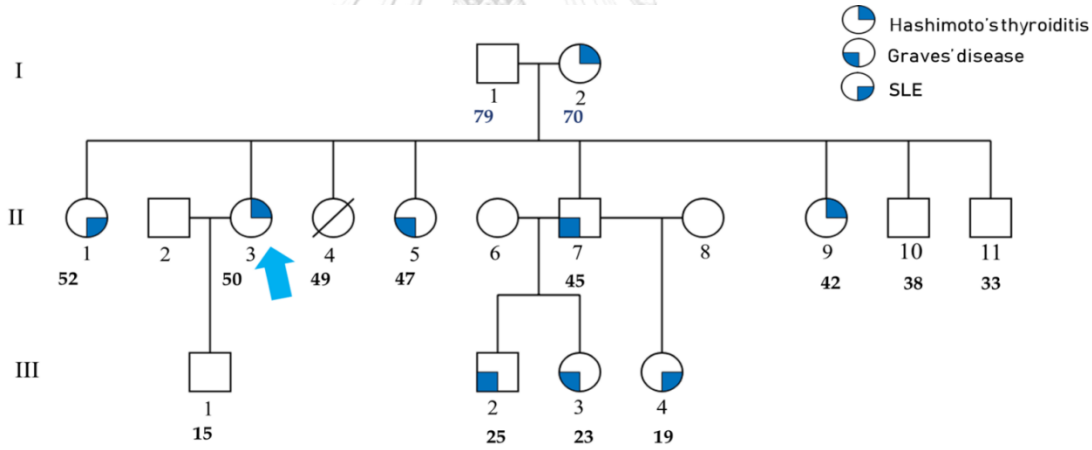


Figure 6.The family pedigree's of proband.

The pedigree represents autoimmune disease is inherited in an autosomal dominant manner.

2. Mutation analysis

2.1 Whole-genome linkage analysis

15 members of family (I-1,I-2,II-1,II-3,II-4,II-5,II-7,II-8,II-9,II-10,II-11,III-1,III-2,III-3 and III-4) were genotyped using Infinium OmniZhongHua-8 BeadChip specific to Chinese populations containing 1,175,489 single-nucleotide polymorphisms (SNPs; Illumina,San Diego, Calif., USA). Parametric linkage analysis was performed by Merlin 1.1.2 software using an autosomal dominant model with the penetrance values being set at 0.6, 0.8, and 0.99.

2.2 Whole-Exome Sequencing

Whole-Exome Sequencing (WES) was performed as described (34). Briefly, genomic DNA was isolated from peripheral blood leukocytes. The DNA sample was prepared as an Illumina sequencing library and in the exome capture step. The sequencing libraries were enriched by SureSelect Human All Exon V7 kits. The captured libraries were sequenced using Illumina NovaSeq 6000 Sequencer. To identify disease-causing variants under the assumption of an autosomal dominant pattern of inheritance. The analysis was made and the following filtering criteria filtered all SNVs and Indels; 1) located in exons or flanking introns of the below-listed genes, 2) not synonymous, 3) rare with 1000G minor allele frequency of less than 1%, 4) <100 in the Exome Aggregation Consortium (ExAC) database, 5) <10 alleles in 1,876 Thai exome controls, 6) (if the variant is a missense) predicted to be damaging by SIFT and Poly Phen, and/or 7) related to the phenotype of the patient. To confirm the presence of the identified variant, we performed PCR and Sanger sequencing in the affected patients and the unaffected patients who

underwent WES. The primer amplified DNA, and the PCR products were treated with Exo-SAP-IT (Affymetrix) followed by Sanger sequencing. Primers used in PCR amplification and sequencing were shown in Table 1.

Table 1. Primer for PCR and sequencing

Primer sequence (5'-3')	Sequences	Ref
LILRB1-long1F	CTGCTCATGACATTGATGCTCTG	Kuroki et al (35)
LILRB1-long1R	CGCTACCATAGTAACAGCGATAC	
LILRB1-long2F	GTATCGCTGTTACTATGGTAGCG	
LILRB1-long2R	ATTACAGGCACTGCCACCACAT	
LILRB1specificF	GTATCGCTGTTACTATGGTAGCG	
LILRB1-1R	GAATTTCTCACCTAGGACCAGGA	

3. Characterize phenotype expression and function of variant from peripheral blood mononuclear cells (PBMC) of familial autoimmune patients.

PBMC were isolated by Ficoll-Hypaque gradient centrifugation (Sigma Chemical Co.) gradient centrifugation. Cells were resuspended in RPMI 1640 culture medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone), 2.0 mM glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin (Sigma-Aldrich) before counting. The PBMC were gently resuspended in a freezing medium containing 90% fetal bovine serum (FBS), 10% dimethyl sulfoxide to give at least 10 million cells/cryovial, depending on the number of PBMC recovered after processing. Aliquots of purified PBMC were cryopreserved and stored in liquid nitrogen until assayed.

3.1 Determination of ILT2 expression levels

We performed quantitative real-time PCR (qRT-PCR) with RNA from PBMC of patients from family, using the TaqMan ® Gene Expression Assay (Applied Biosystems, catalog No. APT2AFH for ILT2, Hs01060665_g1 ACTB, respectively). The ILT2 expression levels were calculated relative to reference genes, ACTB. These were compared with three unaffected controls. Briefly, qRT-PCR reaction composed of 1 ul of cDNA, 10 ul of Luna universal qPCR mastermix (NEB), 8 ul of nuclease-free water, and 1 ul of the probe. All samples were performed in triplicate. GraphPad Prism, 9.1.0 version program, analyzed data. The significant differences were determined using either one-way ANOVA or independent sample t-test as appropriate for group comparisons. Statistical significance was considered at $p\text{-value} < 0.05$.

3.2 To evaluate the expression of ILT2 in different cell populations from the peripheral blood of autoimmune patients and healthy controls.

PBMC from 14 members and five controls were immunostained with an anti-ILT2 conjugated with PE and the following MAB, for T B NK panel use anti-CD4 APC-Cy7 anti-CD8 AF700, anti-CD14 PE-Cy7, anti-CD16 PE-DZ594, anti-CD19 PE-Cy5, anti-CD56 AF647. For DC panel use anti-CD1C PE-DZ594, anti-CD11c AF700, anti-CD123 APC, and anti-CD303 Perch Cy5.5. For the Treg panel, use anti-CD25 APC-Cy7, anti-CD127 PE-Texas Red-A, anti-GARP PE-Cy7, and anti-LAP APC-A. For the Breg panel, use anti-CD19 PerCP-Cy5.5, anti-CD24 PE-Texas Red, anti-CD38 AF700, anti- CD71 PE-Cy7, and anti-CD73

APC. All antibody obtained from BioLegend, San Diego, CA. In addition, an anti-ILT2 MAB (HP-F1) labeled with phycoerythrin (PE, Invitrogen) was also employed. Cells will be analyzed for the expression of ILT2 by FACSAria II (Becton Dickinson, USA).

To study the role of ILT2 in the inhibition of the Treg and Breg subsets by using the HP-F1, an anti-ILT2, a monoclonal antibody (mAb) that mimics the ligand and inhibits the proliferation induced through CD3/TCR. PBMC obtained from all participants was cultured for 48 h in the presence or absence of the HP-F1 anti-ILT2 MAB. The expression on the different lymphocyte subsets was determined by flow cytometry analysis.

3.3 To evaluate the expression and level of cytokine in patient's PBMC

The expressions of IL2, IL 6, IL10, IL12 mRNA in peripheral PBMC of familial autoimmune patients with autoimmune disease were determined by quantitative real-time PCR (qRT-PCR) (StepOnePlus: Applied Biosystems) using the TaqMan ® Gene Expression Assay (Applied biosystems), catalog No. Hs00174114_m1 for IL2, catalog No. Hs00174131_m1 for IL6, catalog No. Hs00961622_m1 for IL10 and Hs01011518_m1 for IL12B.

4. Single-cell RNA sequencing (scRNA-Seq)

We are using scRNA-seq to identify and characterize disease-related immune cell subsets and to identify the transcriptional signatures of immune cells that may be correlated with pathogenicity.

Single-cell RNA sequencing (scRNA-Seq) is a new high-throughput sequencing analysis of the whole transcriptome that aims to measure genes' expression levels in individual cells. Over the past few years, the revolution in scRNA-seq has emerged as the most mature and widely disseminated method in life science research. It can help identify new cell subsets and help uncover the molecular mechanisms of pathogenesis—workflow of single-cell RNA-sequencing (scRNA-seq) experiments as described in figure 7.

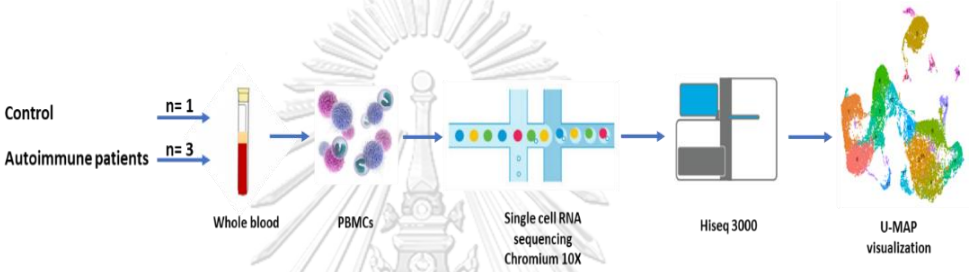


Figure 7.scRNA-seq workflows.

Experimental design showing collection of PBMC from patients and control, with eventual downstream cell isolation, capturing, library preparation, sequencing, and visualization in UMAP.

4.1 Cell isolation, capturing, library preparation and sequencing.

PBMC were isolated using density-gradient centrifugation from heparinized peripheral blood. PBMC were stored at −80 °C in the freezing medium for use. All samples were washed and resuspended in PBS, containing 0.1% BSA. Cell numbers for each sample were counted using an automated cell counter (Countess II, Invitrogen). We assessed the viability of cells in all samples>80%. According to the manufacturer's protocol, the single-cell capturing and downstream library constructions were performed using the Chromium Single Cell 5′ library or 3' v2 library preparation kit (10x Genomics). Briefly, cellular suspensions were partitioned with

barcoded gel beads to generate single-cell gel bead-in-emulsion (GEM), and poly-adenylated transcripts were reverse-transcribed. Full-length cDNA and cell-barcode identifiers were PCR-amplified, and sequencing libraries were prepared and normalized to 3 nM for loading on a HiSeq 3000 (Illumina). With the data of scRNA-seq, unsupervised clustering of cells was performed with R (Seurat package version 2.2). Then the results of unsupervised clustering were visualized by using Uniform Manifold Approximation and Projection (UMAP)-visualization.

4.2 Identification of marker genes and cell-type annotation.

Differential expression of every cluster was calculated using the 'bimod' test as implemented in the Seurat Find Markers function. Genes were found as marker genes with a log2 average differential expression of 0.25 and $P<0.05$.

5. Study the pathogenic effect of the ILT2 variant.

To identify c.479G>A variant in ILT2 is pathogenic for familial autoimmune disease and study the pathomechanism of this mutation. The expression vectors pcDNA3.1+/C-(K)DYK containing the wild-type ILT2 were purchased from GenScript, and the mutant ILT2 vectors of p.G160E (the mutation identified in this study) was generated using Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). Briefly, 1.25 μl 2 × KLD Reaction Buffer, 0.25 μl PCR reaction, 0.75 μl distilled water and 0.25 μl KLD Enzyme Mix were mixed and incubated at room temperature for 5 min and used for the transformation of E. coli DH5alpha with standard techniques. After mutagenesis, the mutant plasmid was verified by sanger

sequencing. The wild-type and mutant were transfected to Jurkat, T lymphocyte cell line without endogenous ILT2 expression using electroporated using AmaxaTM SE Cell Line 4D-NucleofectorTM X Kit S a Lonza 4d strip nucleocuvette (Lonza, Germany), and transfectants were selected in G418-containing medium. ILT2 expression on transfected cells was assessed by real-time PCR and FACS analysis using anti-ILT2 mAb GHI/75 (BioLegend, San Diego, CA).

6. Investigate pathomechanism of ILT2 variant.

Investigate pathomechanism of c.479G>A of ILT2 leads to loss-of-function in this receptor and possible association with autoimmune disease on Jurkat cell line model.

6.1 Determine ILT2 mutation effect on protein expression.

After we transfect wild-type and mutant to Jurkat, total RNAs and proteins will extract from Jurkat with TRIZOL reagent (Invitrogen) and RIPA buffer (Thermo Fisher Scientific), respectively. And will quantify expression by real-time PCR and Western blot analysis, respectively. For Western blot analyses, protein extracted from the cellular lysates of Jurkat cells transfected with ILT2 WT and ILT2 MT expression vectors were prepared. The PierceTM BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

20 ug of protein per sample was separated on 12% sodium dodecyl sulfate-polyacrylamide gels and then transferred by iBlotTM Transfer Stack, PVDF, regular size (Invitrogen, Carlsbad, CA). Monoclonal anti-LILRB1 antibody at 1:500 dilution (cat no. 78144s, Cell Signaling Technology, Danvers, MA) was used as the primary antibody to detect ILT2 WT and MT

proteins, followed by anti-rabbit IgG antibody at 1:2000 dilution (cat no. 7076, Cell Signaling Technology, Danvers, MA) as the secondary antibody. Anti-GAPDH antibody (cat no. Mab1501, Sigma-Aldrich, St Louis, MO) was used as a positive control to determine gel loading equivalency. Results were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo fisher scientific) and chemiluminescence camera (ImageQuant LAS 4000, Amersham)

6.2 To determine phosphorylation level of ILT2 and SHP-1 in ILT2-WT and ILT2-MuT transfect to Jurkat cell line.

Jurkat cells transfected with ILT2 WT and ILT2 MT expression vectors treated with pervanadate (PV) (200 μM sodium orthovanadate and 200 μM H2O2 at 37°C for 5 and 10 min) as indicated were lysed as described above. 20 ug of protein per sample was used to determine the phosphorylation level of ILT2 and SHP-1 by Human Phospho-Immunoreceptor Array (R&D Systems, Catalog # ARY004) following the manufacturer's instructions. Moreover, the phosphorylation level of SHP-1 was also determined by western blot analysis and quantified using the Image J gel analysis program.

7. Statistical analysis

Data were entered into GraphPad Prism, 9.1.0 version program, and analyzed using non-parametric tests. Flow cytometry data for surface expression of ILT2 were evaluated using the Kruskal Wallis test (non-parametric ANOVA). For multiple comparisons, the Dunn test was used.

The Manne Whitney U test analyzed flow cytometry data for surface expression in cell subsets. P < 0.05 was considered statistically significant.

Chapter IV

Results

1. Patients and clinical data

Clinical characteristics of autoimmune disease patients show in table 2.

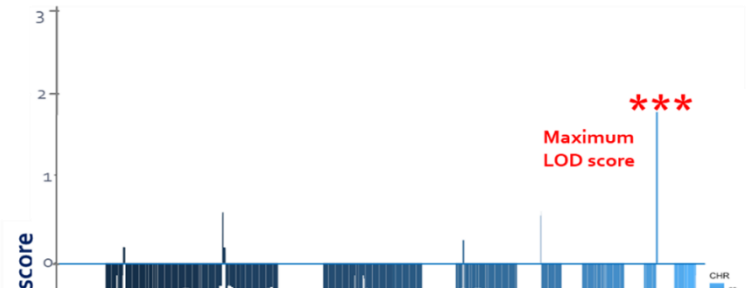
	I-2	II-1	II-3	II-5	II-7	II-9	III-2	III-3	III-4
Clinical	HT	SLE	HT	GD	GD	HT	GD	GD	SLE
Sex	F	F	F	F	M	F	M	F	F
Age	70	52	50	47	45	42	25	23	19
Age on set	67	48	42	42	N/A	33	21	16	16
FT4	1.11 (2559)	N/A	1.18 (2555)	1.77 (2557)	N/A	0.74 (2553)	2.14 (2558)	3.23 (2557)	N/A
TSH	7.8	N/A	4.24	0.006	N/A	25	0.01	0.003	N/A
Anti-thyroglobulin	N/A	N/A	>1,000 (2554)	N/A	N/A	N/A	N/A	N/A	N/A
Anti-thyroid peroxidase	452.3	N/A	>1,000	N/A	N/A	N/A	N/A	N/A	N/A
CRP-QT	N/A	44.800 (2561)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Treatment	Eltroxin	Imuprin Prednisolone Hydroquin	Eltroxin	MMI	Eltroxin	Eltroxin	PTU MMI	MMI	Chloro quine Tacrol imus

Table 2. Clinical characteristics of autoimmune disease patients.

HT= Hashimoto's thyroiditis, GD= Grave disease, SLE = systemic lupus erythematosus, PTU= Propylthiouracil, MMI= Methimazole

2. Mutation analyses

Whole-genome linkage analysis defines the critical region on chromosomes 19 positions 53861258-58379941 size 4.5 Mb with a maximum value of linkage with the LOD score of 2.04 shown as figure 8.



Filtering criteria	Result

Figure 8. Whole-genome linkage analysis (WGL)

WGL identified 1 region (star) on chromosome 19 to be significantly linked to disease in this pedigree.

With the assumption of an autosomal dominant model, whole-exome sequencing results were analyzed with the filtering steps mentioned above and revealed only one DNA candidate variant. A heterozygous missense c.479G>A variant (chr19:55143506, p.(Gly160Glu), rs866926837, [G/A]AGAAGATGAACACCCACAATG, forward strand) in the *ILT2* gene was identified in the patient, located within the linked region on chromosome 19. This variant has not been identified in the ExAC but was found in 1/1876 individuals in the in-house Thai Exome database. Therefore, the prediction programs, SIFT/Polyphen, are inconclusive. This variant has never been previously reported in patients.

Total number of variants after variants calling (1000 Genomes Project Consortium, dbSNPs)	215364
Select variants found in all 9 affected individuals	2518
After exclusion of variants not in or close to the coding regions	571
After variant annotation and filtering with exclusion of variants with coverage <10x; minor allele frequency < 1% in the 1000 Genomes Project and EXAC; and non-coding variants and synonymous exonic variants	11
After exclusion of variants found in an in-house database of 1,876 Thai exomes	3
Located in the identified linked regions	1
Gene	*LILRB1 (ILT2)*
Variant	
Coordinate	chr19:55143506
Genotype	Heterozygous
Variant	c.479G>A
Protein	p.(Gly160Glu)
SIFT	deleterious (0.01)
PolyPhen-2	Possibly damage (0.658)

Table 3. Filtering criteria for the exome sequencing of autoimmune patients.

Sanger sequencing confirmed the novel c.479G>A was detected in all 9 available affected patients and 3 in unaffected family members (Figure 9), suggesting its pathogenicity. Mutation analyses were demonstrated in Tables 3 and pedigree showed in figure 10.

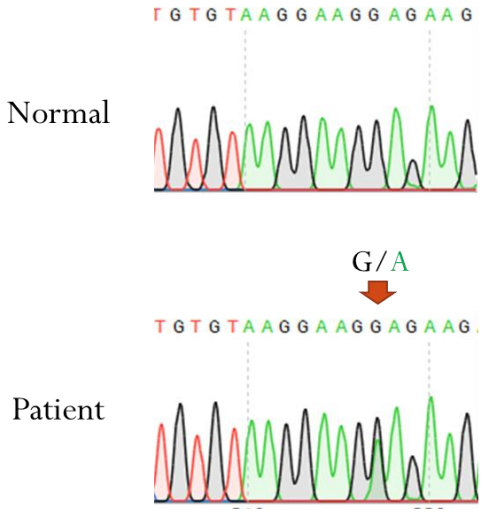


Figure 9. Chromatograms show mutation in *ILT2* exon 5 of affected patients.

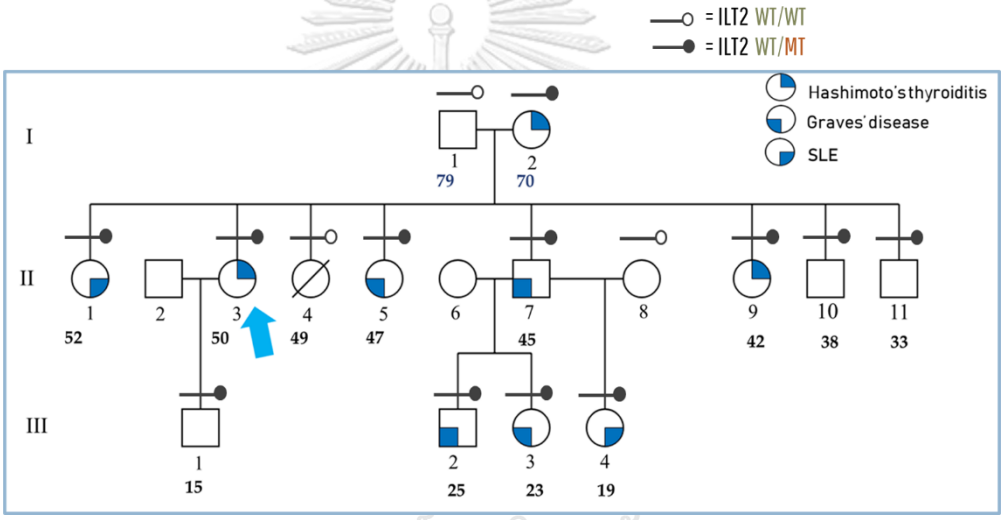


Figure 10. The pedigree of familial autoimmune disease with the *ILT2* variant.

To quantify ILT2 expression levels, we performed quantitative real-time PCR (qRT-PCR) with RNA from PBMC of patients. The expression levels of ILT2 were calculated relative to a reference gene, ACTB. The results revealed no difference in mRNA expression in the affected individual compared with the 2 unaffected controls. However, the RNA of 1 Hashimoto's thyroiditis patient cannot be determined.

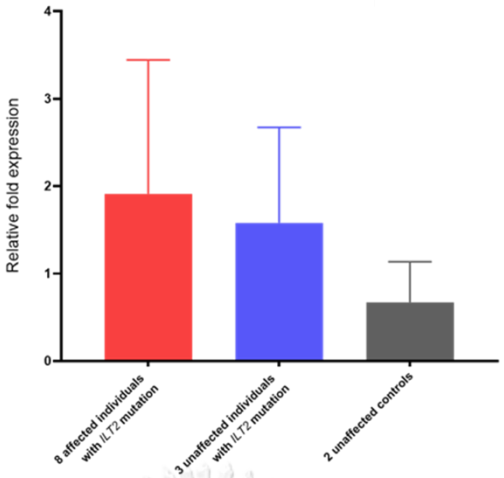


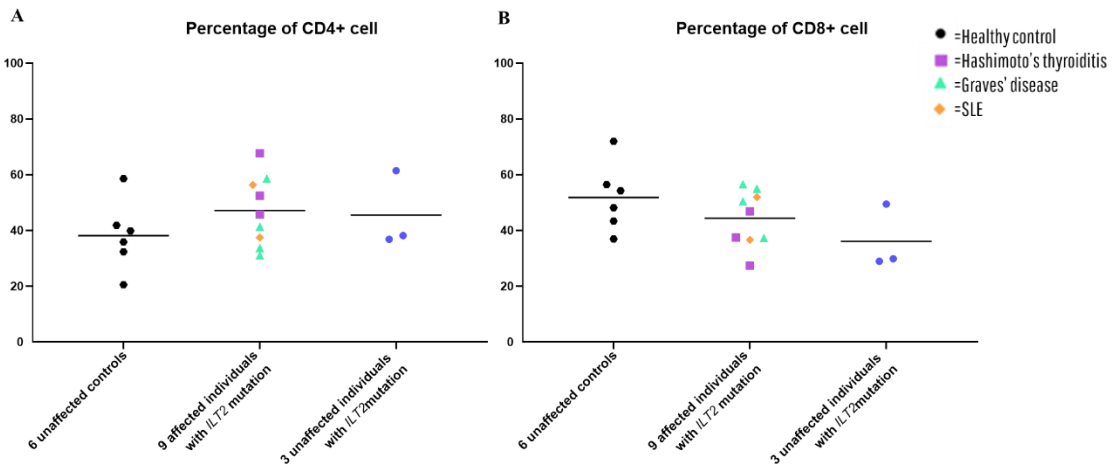
Figure 11. qRT-PCR study mRNA expression of *ILT2* in PBMC.

qRT-PCR shows no appearance of different mRNA expression in 8 autoimmune patients with *ILT2* mutation compared with 3 unaffected family members with mutation and 2 unaffected controls. *ILT2* expression was normalized by ACTB.

3. Phenotype expression and function of variant from PBMC of familial autoimmune patients

Percentage of T, B, NK, monocyte and dendritic cell of familial autoimmune patients

PBMC from autoimmune disease patients (n=12) showed not difference in percent of CD4+ lymphocytes, CD8+ lymphocytes, CD56+, CD19+, monocyte, myeloid dendritic cell, plasmacytoid dendritic cell compared with controls (n=6) as shown in figure 12.



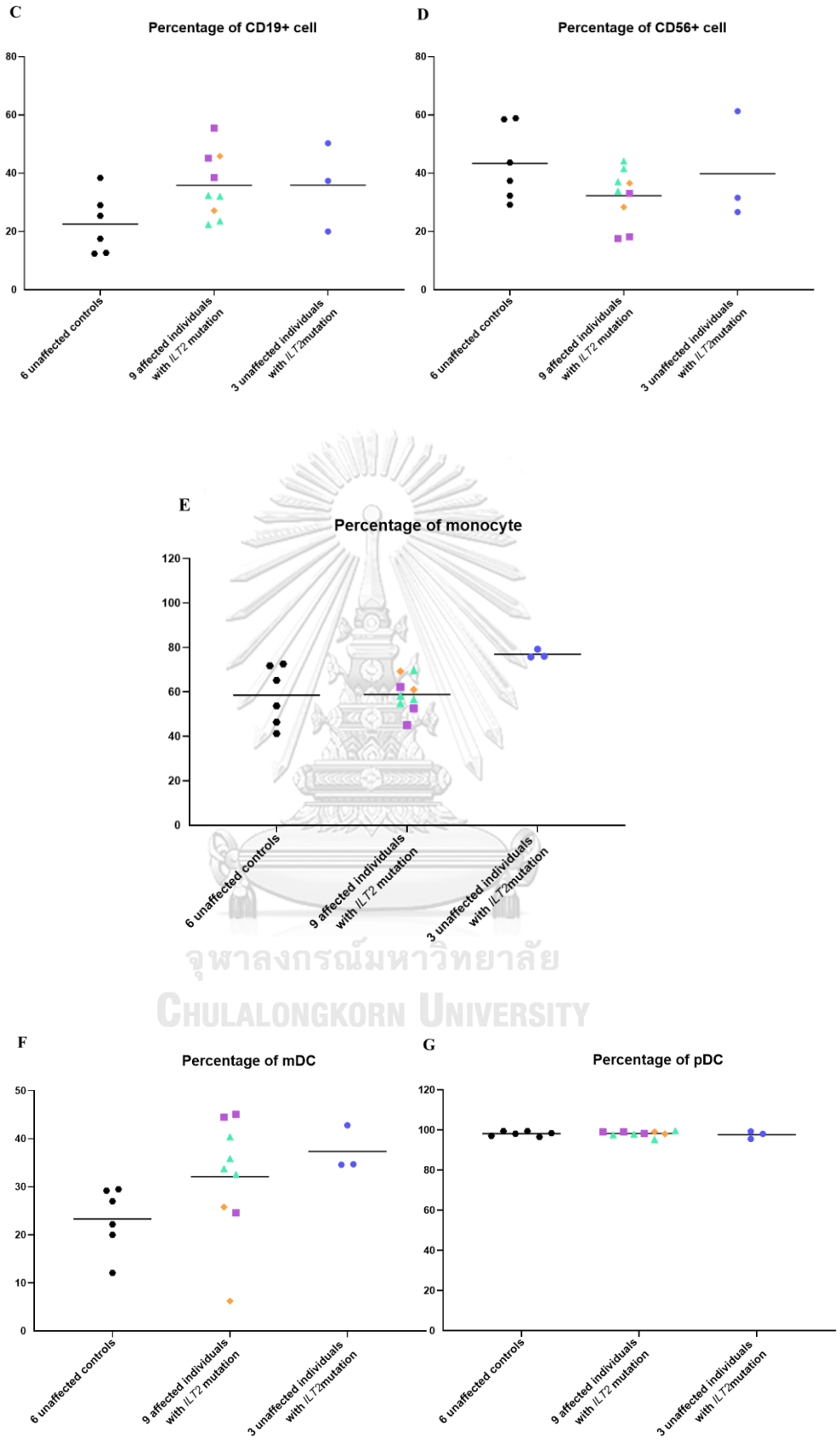
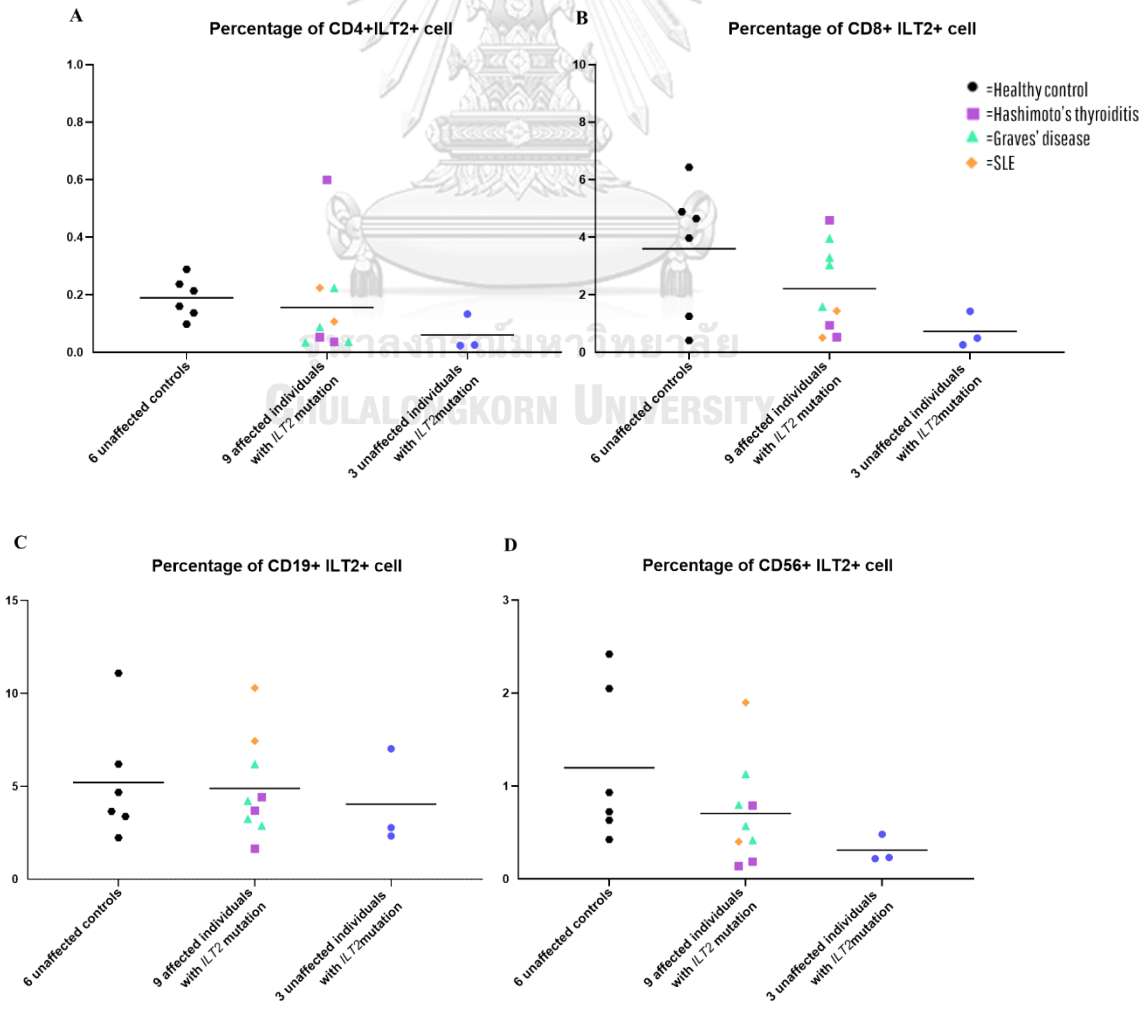


Figure 12. Percentage of PBMC from familial autoimmune patients.

9 affected individuals who have *ILT2* mutation, 3 unaffected individuals who have *ILT2* mutation and 6 unaffected controls were analyzed by flow cytometry in CD4+, CD8+, NK cell, B cell, monocyte, mDCs and pDCs, respectively (A-G).

Percentage of *ILT2* + cell from T, B, NK and dendritic cell of familial autoimmune patients

This work aimed to study the expression of the inhibitory receptor ILT2 in T, B, NK, and dendritic cells populations from peripheral blood mononuclear cells (PBMC) from patients with a mutation in ILT2 autoimmune disease individuals. No significant differences in expression of the inhibitory receptors ILT2 in CD4+, CD8+, CD19+, CD56+, and dendritic cell compared with control.



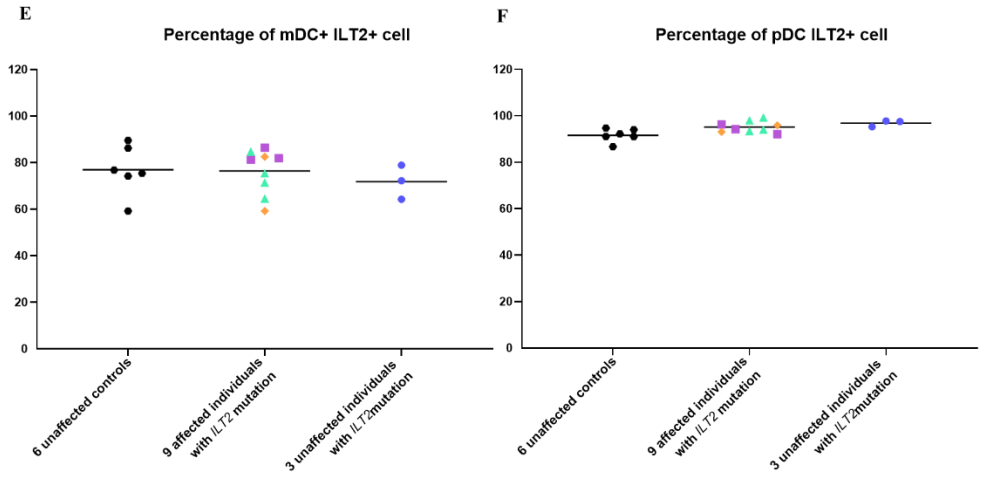


Figure 13. Percentage of *ILT2* + cell from PBMC of familial autoimmune patients

Percentage of PBMC from 9 autoimmune patients who have ILT2 mutation and three unaffected who have the mutation, and 6 unaffected controls were analyzed by flow cytometry for the expression of ILT2 in each immune cell. (A-F)

Percentage of Breg and *ILT2* + Breg cell in familial autoimmune patients

For the Breg panel, we first analyzed the expression of ILT2 in transitional B cell, plasmablast, and Breg cell populations from the peripheral blood of autoimmune disease patients and healthy subjects and study the effect of anti-ILT2 HP-F1 and ILT2 expression of Breg panel.

We found the percentage of transitional B (CD19+CD38hiCD24hi) was not different as well as plasmablast (CD19+CD38hiCD24-) and Breg cell (CD19+CD73-CD25+CD71+) when compared with controls. Interestingly, we found a significantly higher level of plasmablast of 9 affected individuals (P=0.0387) and 3 unaffected who have a mutation in ILT2 (P=0.033) when treating with HP-F1 (Figure 14).

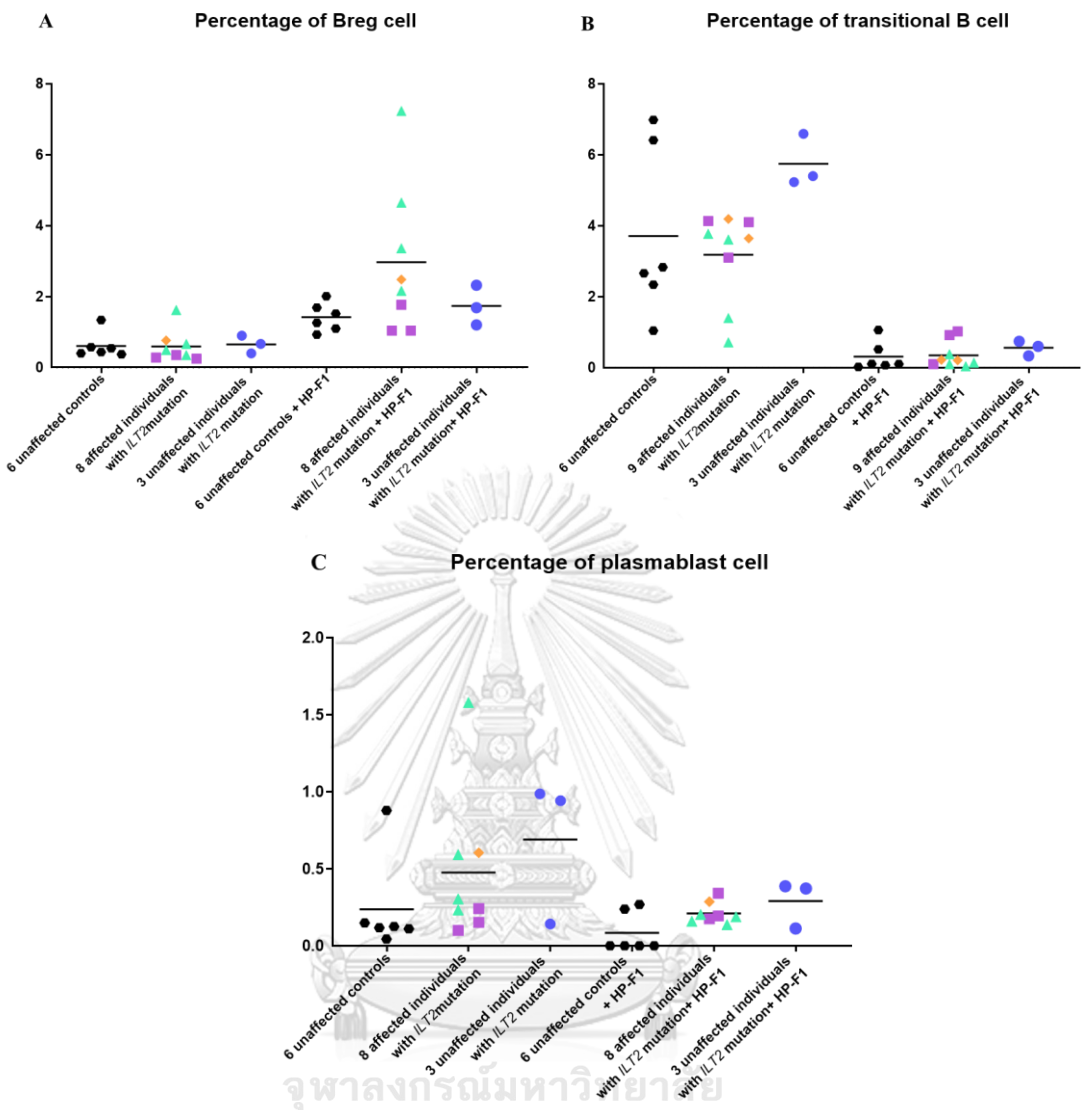


Figure 14. Percentage of Breg panel from PBMC in familial autoimmune patients

Percentage of transitional B cell, plasmablast, and the Breg from 8 affected individuals, 3 unaffected individuals with the mutation, and 6 unaffected controls were analyzed by flow cytometry (A-C).Data correspond to the arithmetic mean and s.d. *P<0.05, **P<0.01, ANOVA, or Kruskal–Wallis test.

However, no significant differences in expression of ILT2 in the Breg, transitional B cell, plasmablast from patients compared with controls (Figure 15)

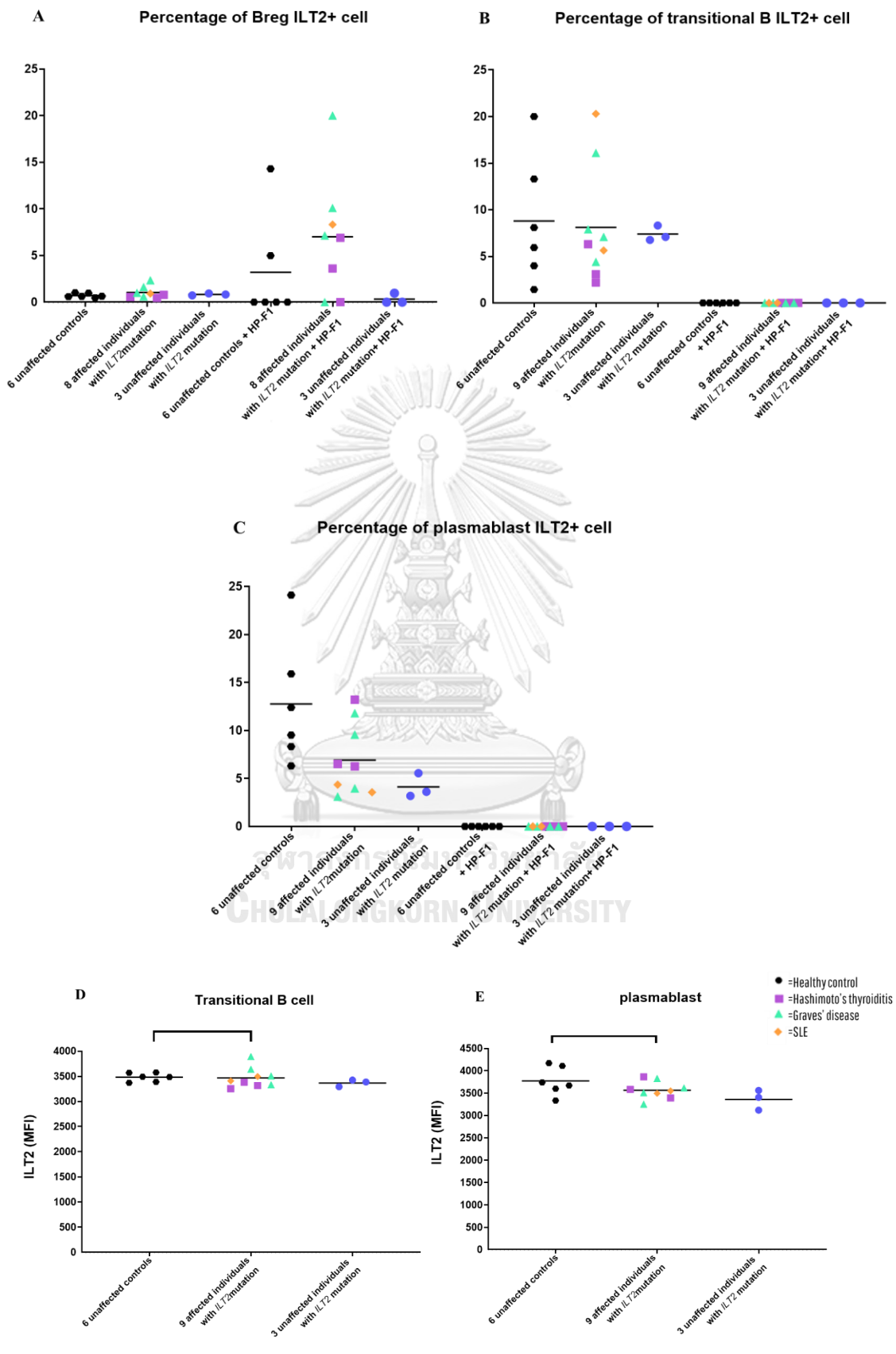
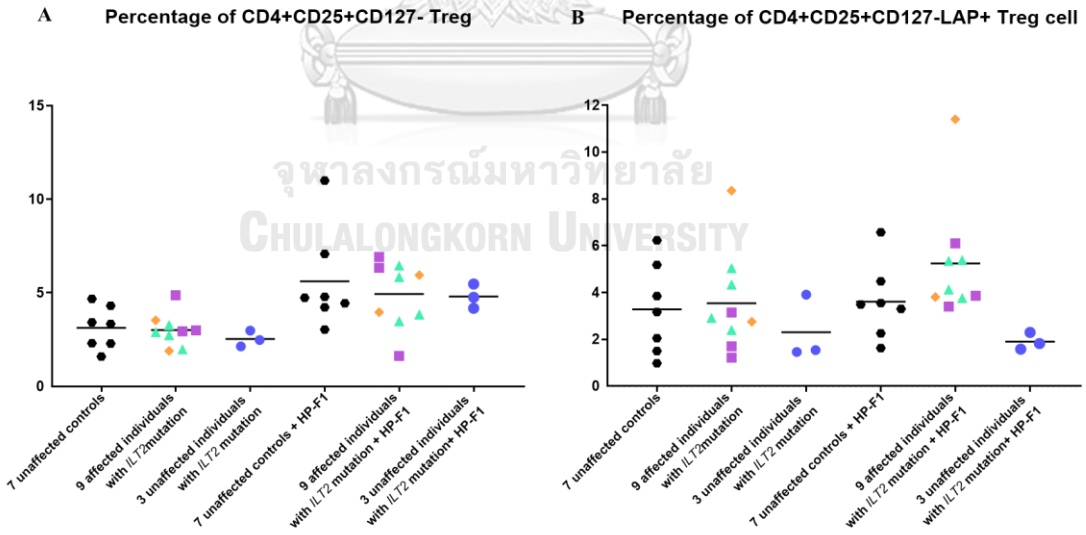

A
Percentage of Breg ILT2+ cell
B
Percentage of transitional B ILT2+ cell
C
Percentage of plasmablast ILT2+ cell
6 unaffected controls
8 affected individuals with ILT2mutation
3 unaffected individuals with ILT2 mutation
6 unaffected controls + HP-F1
8 affected individuals with ILT2 mutation + HP-F1
3 unaffected individuals with ILT2 mutation+ HP-F1
9 affected individuals with ILT2mutation
9 affected individuals with ILT2 mutation + HP-F1
3 unaffected individuals with ILT2 mutation+ HP-F1
D
Transitional B cell
E
plasmablast
ILT2 (MFI)
=Healthy control
=Hashimoto's thyroiditis
=Graves' disease
=SLE

Figure 15. Evaluation of *ILT2* expression on Breg subset in family of autoimmune disease. Percentage of PBMC from 9 autoimmune patients who have ILT2 mutation and three unaffected who have the mutation and 6 unaffected controls were analyzed by flow cytometry for the expression of ILT2 in the Breg subset. (A-D). MFI of ILT2 expression in transitional B cell (E) and MFI of ILT2 expression in plasmablast (F)

Percentage of Treg subset and *ILT2*+ Treg subset in familial autoimmune patients

We characterize a subset of CD4+CD25+CD127- Treg that express latency-associated peptide (LAP) on their cell surface CD4+CD25+CD127- LAP+ cells. In addition, we measured the combined LAP+ and GARP+ in CD4+CD25+CD127- Treg cells to estimate the proportion of Tregs. However, we found the percentage of Treg (CD4+CD25+CD127-LAP+GARP+) was not different in autoimmune patients compared with controls (Figure 16).



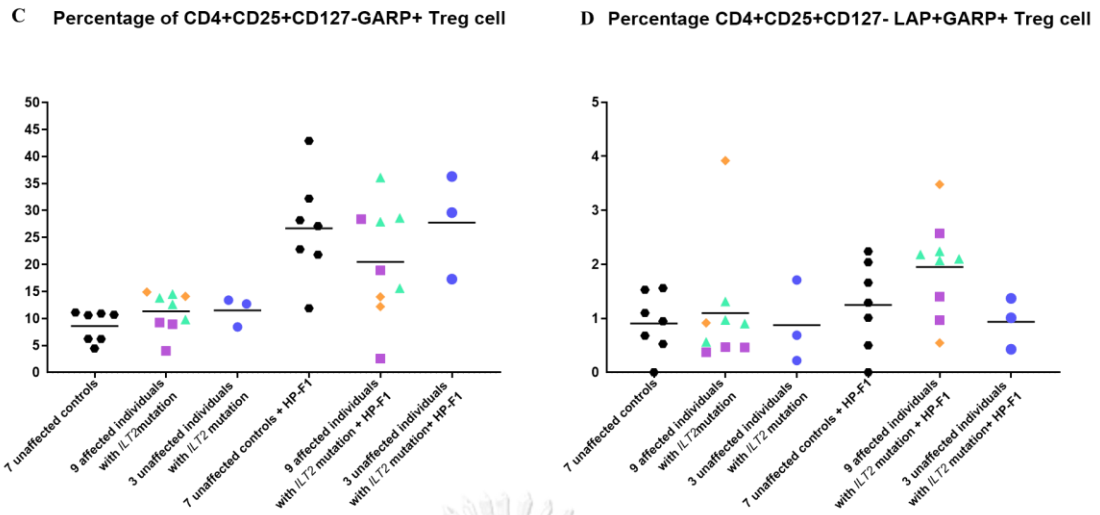
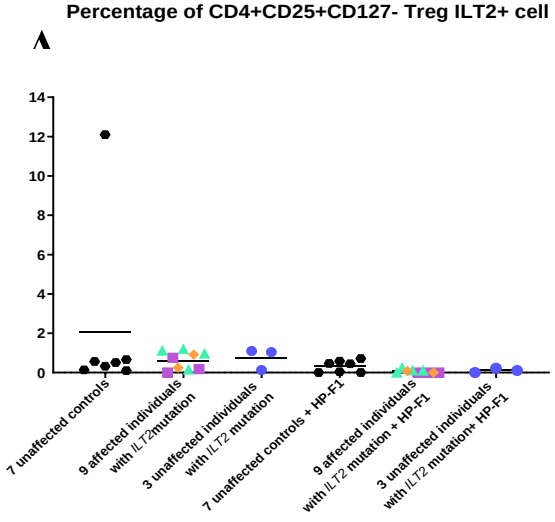


Figure 16. Percentage of PBMC in Treg panel

Percentage of PBMC in Treg panel from autoimmune patients, unaffected individuals with ILT2 mutation, and unaffected controls were analyzed by flow cytometry in Treg panel (A-D).

For the Treg panel, we found We observed a significantly lower level of MFI LAP+ ILT2+ in all autoimmune disease patients (P=0.0072) and when activated with HP-F1 (P=0.0043). Besides, We observed a significantly lower level MFI GARP+ ILT2+ (P=0.0037) without activation with HP-F1 in all autoimmune disease patients. Finally, we found a significantly lower MFI ILT2+ in LAP+ GARP+ in all autoimmune diseases when compared with control (P=0.0293). However, not difference appear in Treg ILT2+ was detected in all individuals (Figure 17).



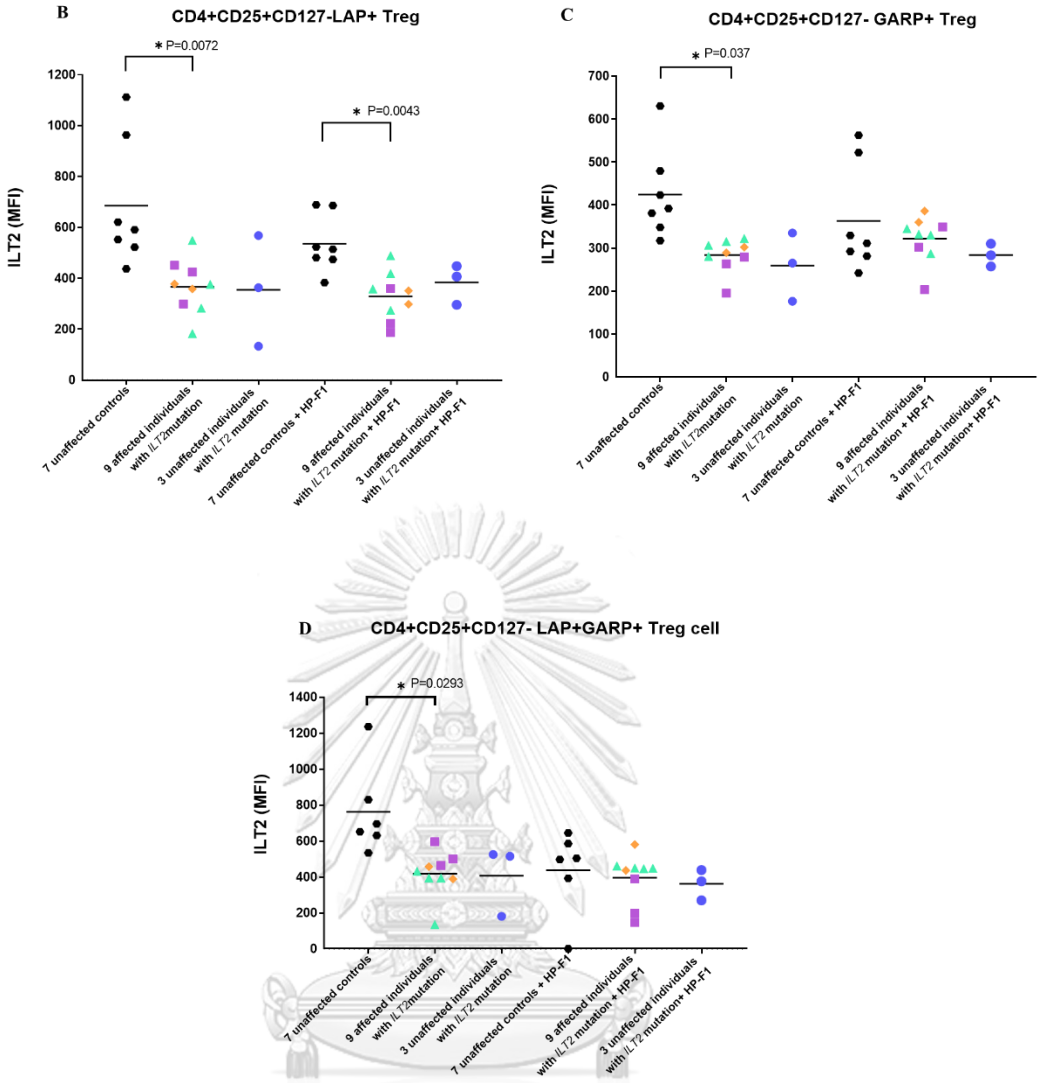


Figure 17. Evaluation of *ILT2* expression on Treg panel in family of autoimmune disease. Percentage of PBMC from autoimmune patients who have ILT2 mutation (n=9) and unaffected who have the mutation (n=3) and unaffected controls (n=6) were analyzed by flow cytometry for the expression of ILT2 in each immune cells (A). MFI of ILT2 expression in LAP+ Treg cells (B), MFI of ILT2 expression in GARP+ Treg cells (C), and MFI of ILT2 expression in LAP+GARP+ Treg cells (D)

To evaluate the expression and level of cytokine in patient's PBMC

The results reveal no difference in the expression and level of IL2, IL6, IL10 and IL12B in 8 affected, 3 unaffected with mutation compared to 2 family members who do not have the mutation (unaffected control).

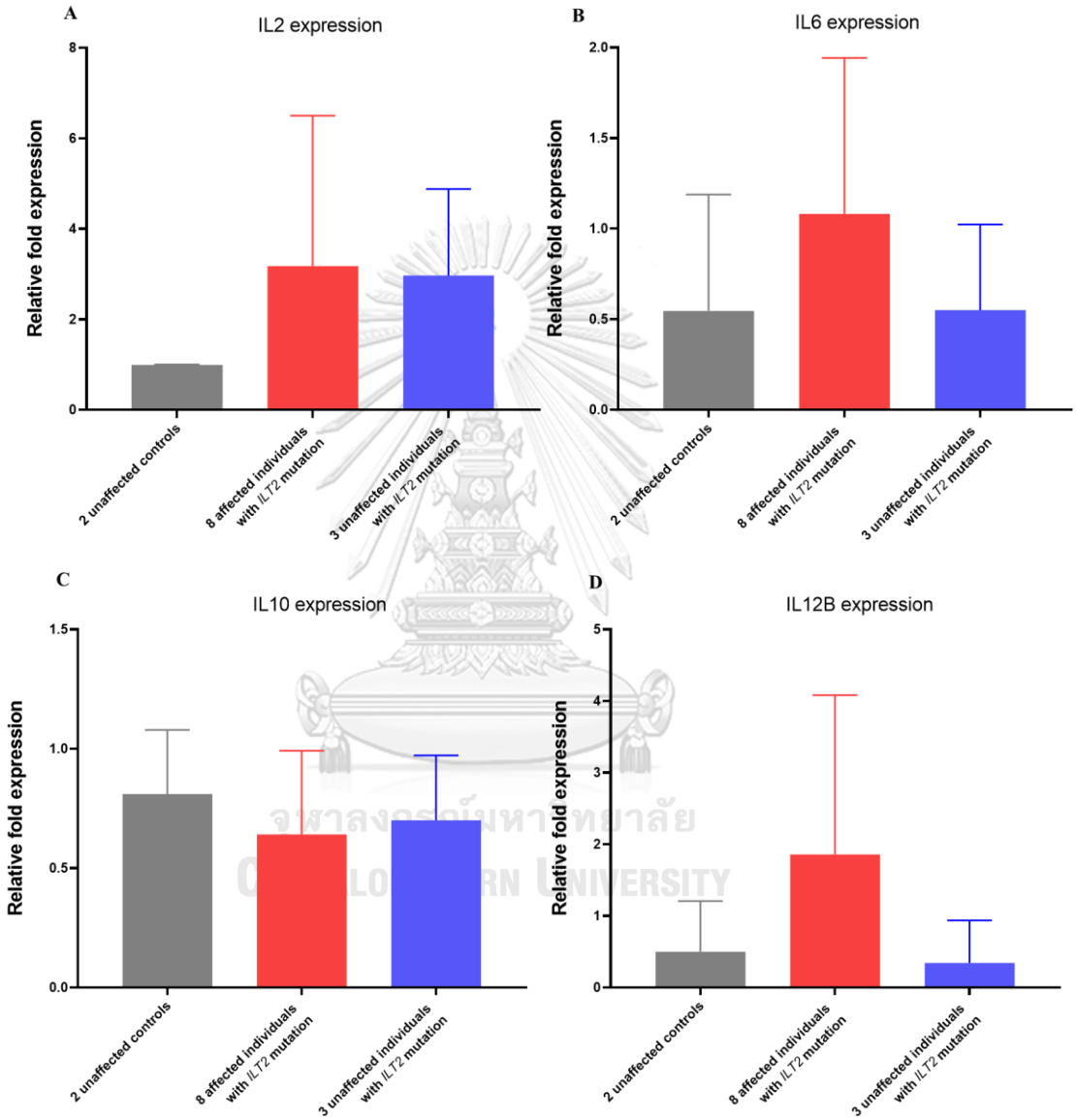


Figure 18. qRT-PCR to study mRNA expression of IL2, IL6, IL10 and IL12, in patients' PBMC.

qRT-PCR shows no appearance of different IL2, IL6, IL10, and IL12 mRNA expression in 8 affected individuals with ILT2 mutation than 3 unaffected individuals with ILT2 mutation and 2 unaffected controls. ACTB normalized IL2, IL6, IL10, and IL12 expression.

Single-cell transcriptome profiling of PBMC identify and characterize disease-related immune cell subsets and found the transcriptional signatures of immune cells that may be correlated with pathogenicity.

A total of 3 autoimmune disease patients composed of 2 are autoimmune thyroid disease (AITD), 1 with Hashimoto's thyroiditis (HT), 1 with Graves disease (GD) and 1 is systemic lupus erythematosus (SLE), and 1 healthy subject with similar gender and age to individuals were studied as controls. Clinical characteristics of autoimmune disease individuals show in table 4.

	Patient 1	Patient 2	Patient 3
Clinical	HT	GD	SLE
Sex	F	F	F
Age	42	23	19
Age on set	33	16	16
FT4	0.76 (2010)	3.23 (2014)	N/A
TSH	14.6	0.003	N/A
Treatment	Eltroxin	MMI	Chloroquine Tacrolimus

Table 4.Clinical characteristics of autoimmune disease individuals.

To characterize the cell types and gene expression profiles of PBMC in autoimmune patients, we performed scRNA-seq of patient PBMC compared to sex-matched control PBMC. First, the PBMC were isolated from 3 patients and 1 control, and then these samples were analyzed by single-cell RNA sequencing technology (10X Genomics Chromium). The total

numbers of recovered cells were 39,759, including 12,673 for controls and 27,086 for autoimmune patients.

PBMC was clustered for 20 cellular compositions visualized by Uniform Manifold Approximation and Projection (UMAP) based on expression profiles.

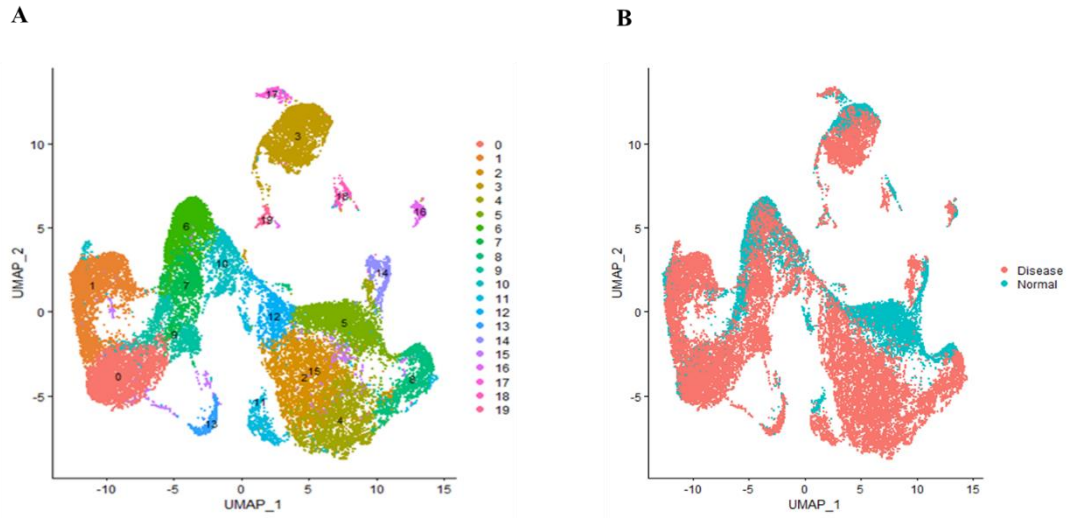


Figure 19. U-MAP visualization of 20 clusters

a) combined all samples b) separate patients(red) and control(blue)

Twenty clusters representing distinct cell populations were identified (Figure 19). We used the characteristic marker gene expression to assign cellular identities to each cluster (Table 1). Several distinct lymphoid cell clusters, including CD8+ T cells (CD8A), CD4+ T cells (IL7R), NK cells (GNLY, NKG7), and B cells (MS4A1), could be discriminated in PBMC. Myeloid populations were also identified, including CD14+ (CD14, LYZ) and CD16+ monocytes (FCGR3A), and 2 distinct groups of DCs—plasmacytoid (pDCs) (IL3RA, CLEC4C) and conventional (cDCs) (CD1C, CST3). For platelet, we defined this cluster according to the expression of canonical gene markers of PPBP (Table 5).

Table 5.Conical marker that used to define each cluster.

Cluster ID	Marker	Cell type
0	CD8A	CD8+ T cell
1	GNLY, NKG7	NK cell
2	CD14,LYZ	CD14+ monocyte (1)
3	MS4A1	B cell (1)
4	CD14,LYZ	CD14+ monocyte (2)
5	CD14,LYZ	CD14+ monocyte (3)
6	IL7R,CCR7	Naïve CD4+ T cell (1)
7	IL7R,CCR7	Naïve CD4+ T cell (2)
8	FCGR3A,MS4A7	FCGR3A+ monocyte
9	IL7R	CD4+ T cell
10	MT-ND4L	Basophil
11	S100A8,S100A9	CD1C- CD141- dendritic cell
12	CD14,LYZ	CD14+ monocyte (4)
13	PPBP	platelet (1)
14	FCER1A,CST3,CD1C	myeloid/conventional dendritic cells 2
15	PPBP	platelet (2)
16	FCER1A,CST3,IL3RA,CLEC4C	plasmacytoid dendritic cell
17	MS4A1,CD27	memory B cell
18	MS4A1	B cell (2)
19	TYMS	Treg

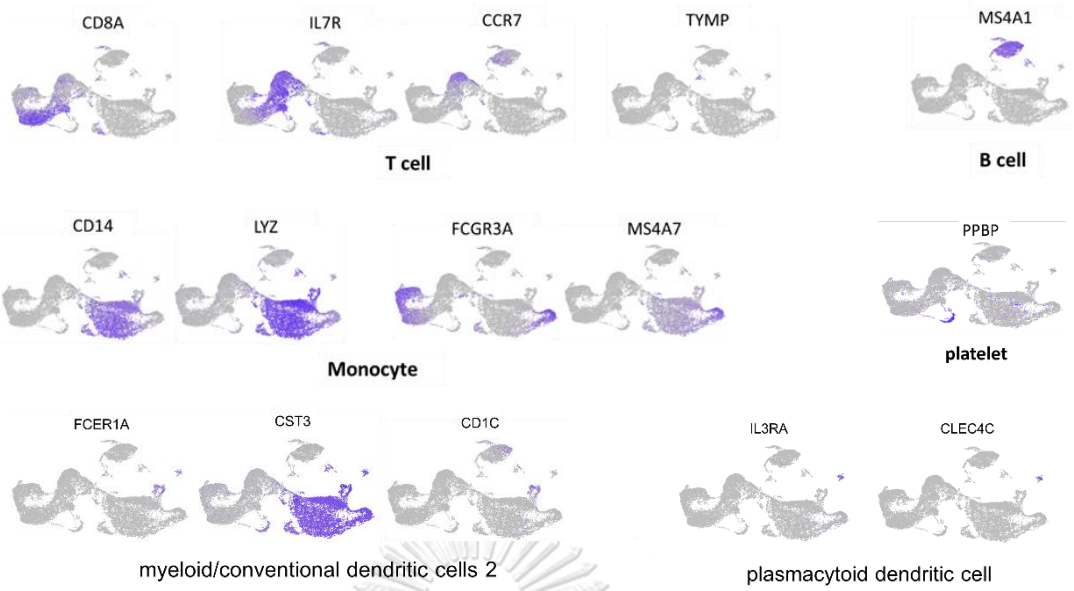


Figure 20.Feature plot of expression of conical marker genes used to define all the cell types.

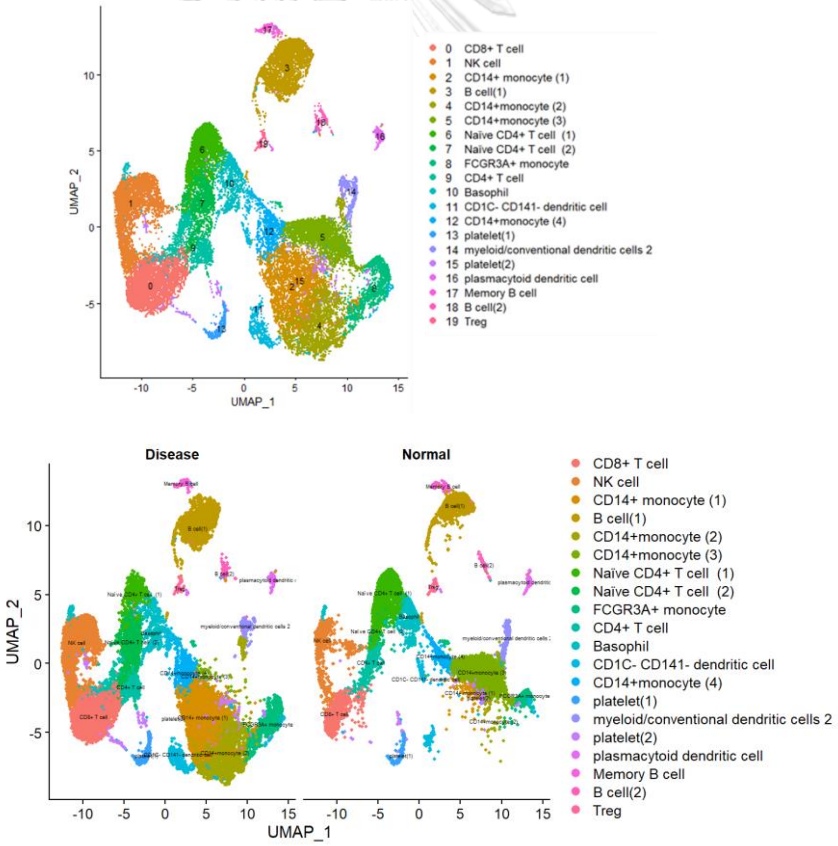


Figure 21. High-dimensional transcriptomic scRNA-seq clustering reveals distinct cell type subpopulations.

20 clusters across 37,810 cells from all cell types on a UMAP visualization (no. of patients=3, control =1) a) all samples b) compare between patients and control.

Monocytes are the largest cell population in patients and different cell percentages in autoimmune disease patients.

We observed monocytes are the largest cell population, which were clustered into 5 clusters, CD14+ monocyte (1), CD14+ monocyte (2) , CD14+ monocyte (3), CD14+ monocyte (4) and FCGR3A+ monocyte. The marker of each cluster was show in table 6.

Cluster 2 =monocyte (1)	Cluster 4= monocyte (2)	Cluster 5 = monocyte (3)	Cluster 12 = monocyte (4)	Cluster 8 = FCGR3A+ monocyte
S100A8	APOBEC3A	LYZ	NEAT1	LYPD2
FOS	TYMP	S100A9	S100A8	LST1
S100A9	FOS	LGALS2	CSF3R	IFITM3
S100A12	EGR1	MNDA	FOSB	MS4A7
CEBPD	CEBPD	S100A8	LINC00936	SERPINA1
TYMP	CD14	S100A12	SLC11A1	FCGR3A
RGS2	IFITM3	VCAN	NAMPT	RP11-290F20.3
G0S2	SPI1	TKT	FOS	AIF1
LINC00936	SAT1	FCN1	VCAN	CDKN1C
EGR1	FCN1	LGALS3	RGS2	WARS

Table 6. Top 10 marker of each monocyte cluster

All CD14+monocyte clusters not only contained validated markers reported in previous studies such as CD14, LYZ, S100A12, S100A8, S100A9, VCAN, and MNDA (36) but also identified several markers such as LGALS1, LGALS2. However, each monocyte cluster has a different expression gene signature. CD14+ monocyte (1) expressed classical monocyte marker genes, whereas CD14+ monocyte (2) distinctively expressed a gene signature (e.g., APOBEC3A,TYMP, IFITM3,SPI1, SAT1) involved in cellular development and differentiation,

the catabolic pathway of polyamine metabolism and most crucial transcription factor in Macrophage and monocyte development. Moreover, CD14+ monocyte (3) express LGALS3, TKT, FCN1 involved leukocyte degranulation, purine nucleoside triphosphate metabolic process, and regulation of cytokine production and CD14+ monocyte (4) express NEAT1, CSF3R and NAMPT related immune response-regulating signaling pathway, phagosome, and oxidative phosphorylation. For FCGR3A+ monocyte or CD16+ monocyte express conical marker FCGR3A+

We found cluster 4 CD14+ monocyte (2), cluster 2 CD14+ monocyte (1), and Naïve CD4+ T cell (2) of patients have a higher cell number than control, respectively.

Moreover, we further delineate M1 and M2 macrophage in each monocyte cluster using expression of M1 and M2 macrophage marker and found CD14 monocyte (2) likely M2 macrophage ($p<0.05$) while CD14 monocyte (4) likely M1 macrophage ($p<0.05$) using list marker from a previous study (37).

4. Pathogenicity of the *ILT2* missense variant, p.G160E

Site-directed-mutagenesis of p.cDNA 3.1+/c-(k)-DYK- LILRB1 was generated by Q5® Site-Directed Mutagenesis Kit (NEB, England) transfect Jurkat cell line that does not has endogenous ILT2. Wild-type and plasmid mutant were extracted and validate sequence by sanger sequencing, as shown in Figure 22.

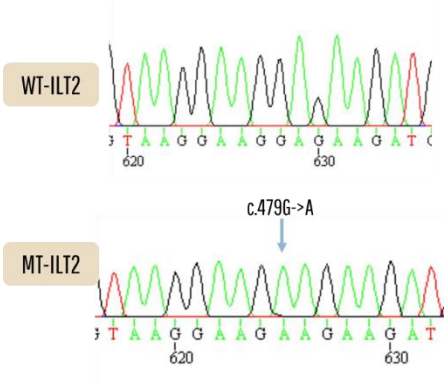


Figure 22 .Chromatograms of the wild-type and mutant *ILT2* extracted from transfected jurkat cell line.

Sanger sequencing shows G to A substitution in *ILT2* gene of the mutant.

To identify *ILT2* expression on transfected cells

Real-time PCR reveals the *ILT2* expression of the wild-type and mutant *ILT2* at 46 days and 52 days of transfection.

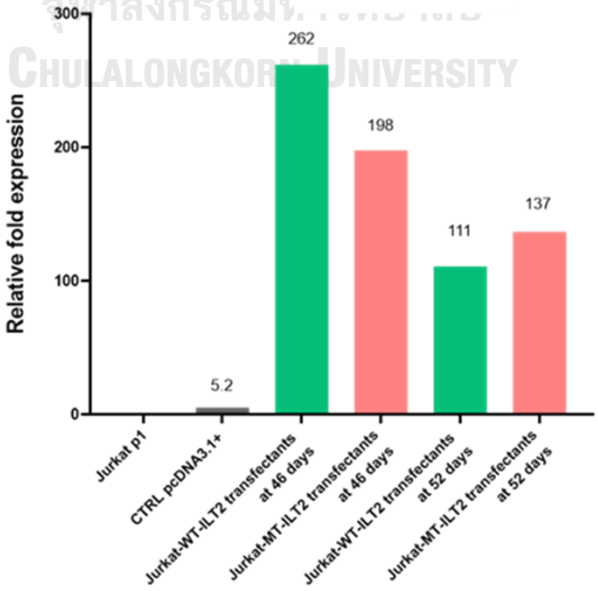


Figure 23. qRT-PCR reveal level of *ILT2* expression from jurkat-*ILT2* transfectants.

Moreover, FACS analysis showed cell surface expression of *ILT2* in transfected jurkat with WT-*ILT2* and MT-*ILT2*

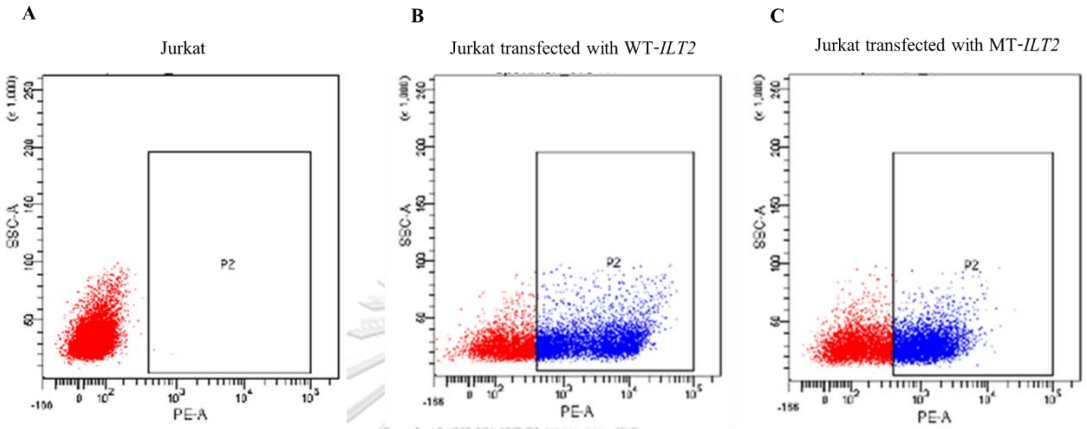


Figure 24. *ILT2* membrane expression, evaluated by flow cytometry.

ILT2 expression on transfected cells was assessed by FACS analysis using anti-*ILT2* mAb GHI/75 in jurkat (a), jurkat transfected with WT-*ILT2* (b), and jurkat transfected with MT-*ILT2* (c)

To determine *ILT2* mutation effect on protein expression

Western blotting showed that detected the wild-type and mutant ILT2 proteins at similar sizes of the expected size (110 kDa). In addition, the level of expression of the mutant was similar to wild-type.

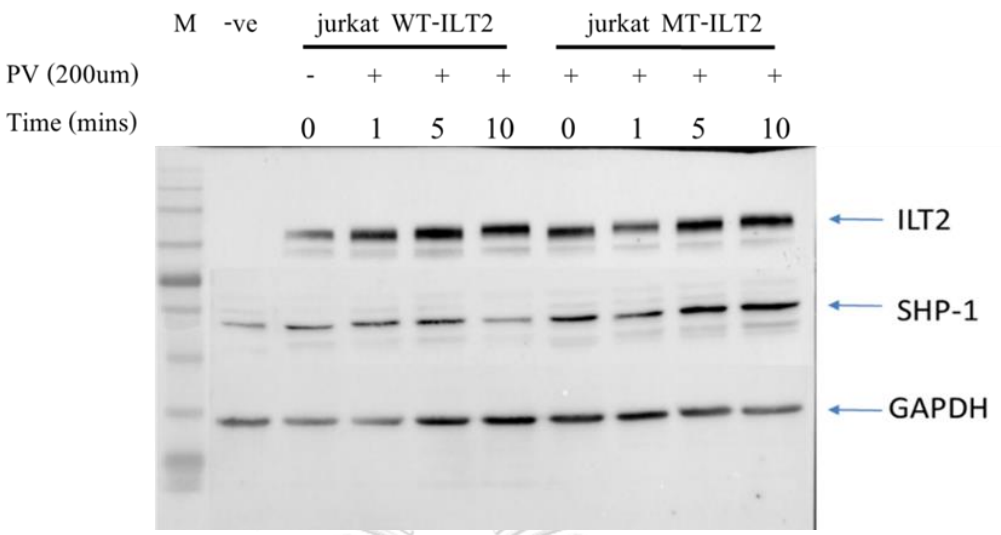
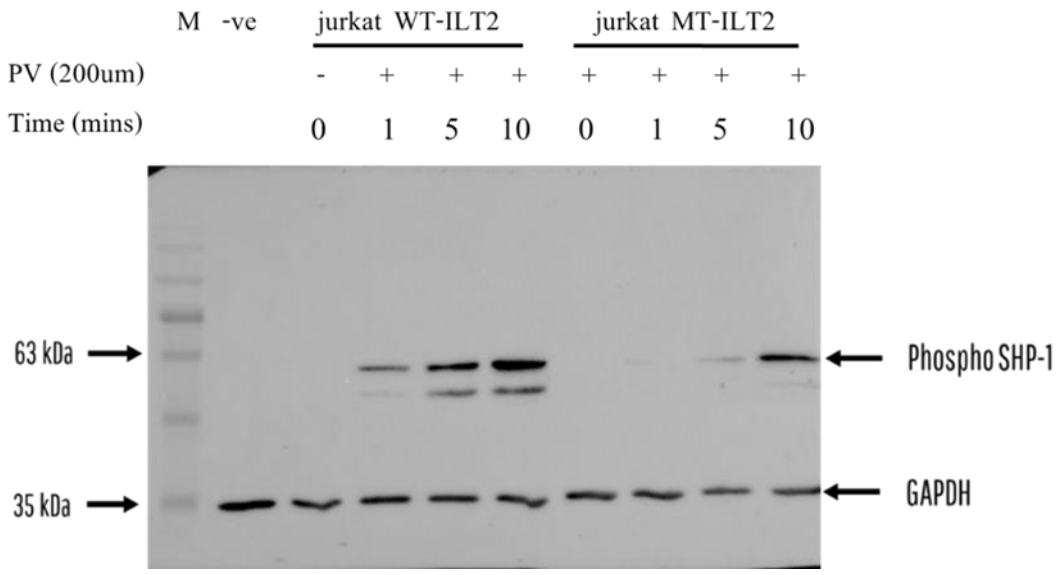


Figure 25.The level of expression of *ILT2* and *SHP-1* from *ILT2*-WT transfected jurkat cells and *ILT2*-MT transfected jurkat cells.

To determine phosphorylation level of ILT2 and SHP-1 in ILT2-WT and ILT2-MT transfect-Jurkat cell line

PV treatment induced substantial tyrosine phosphorylation of ILT2, we found phosphorylation level of SHP-1 was decreasing in ILT2-MT transfect-Jurkat cell at 1,5 and 10 mins.



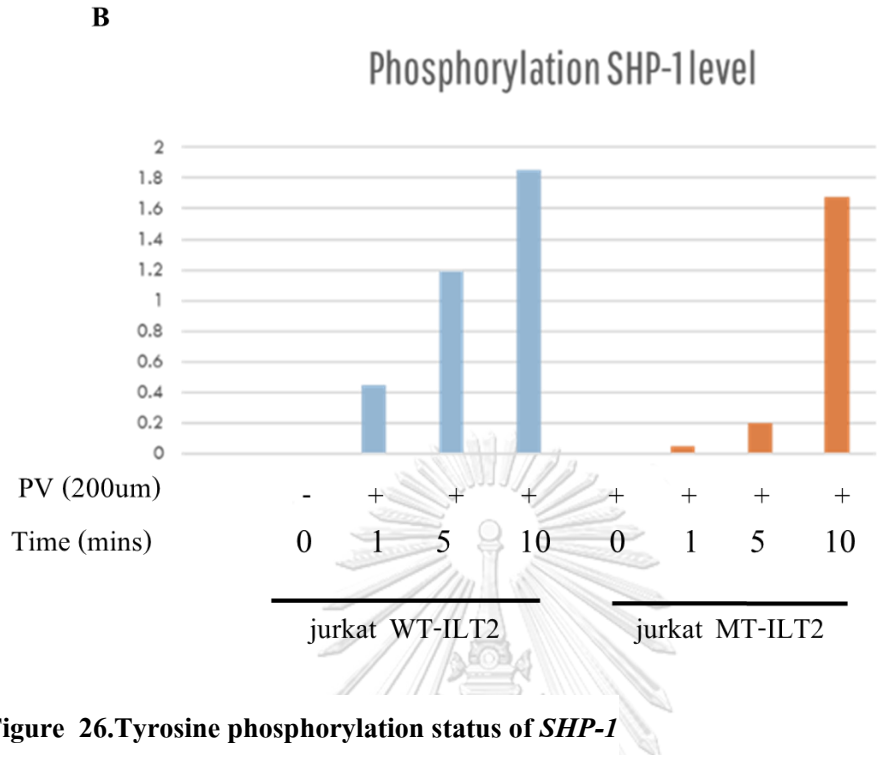
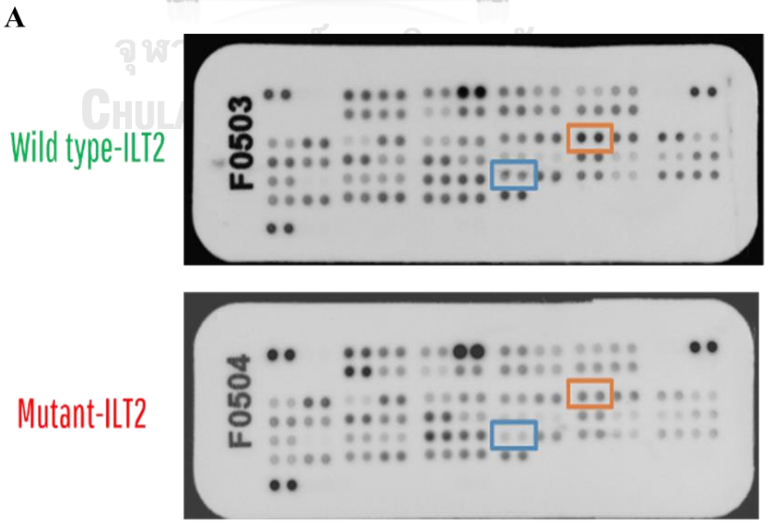


Figure 26.Tyrosine phosphorylation status of *SHP-1*

The tyrosine phosphorylation status of SHP-1 was examined using Western blot analysis. ILT2-WT and ILT2-MT transfect-Jurkat cell lines treated with PV were lysed (A). Representative western blot graphs showing phosphorylation level (B)



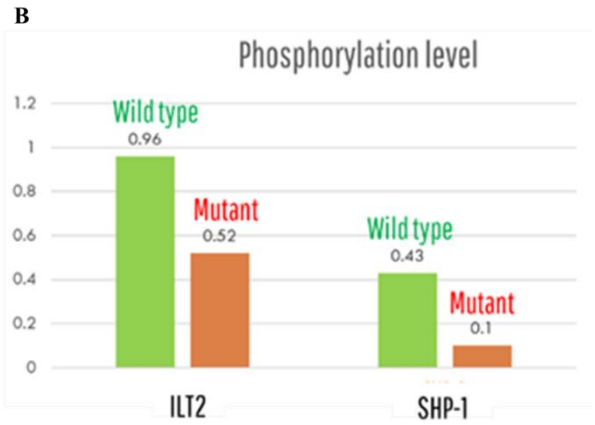


Figure 27. The tyrosine phosphorylation status of *ILT2* receptor and *SHP-1* was examined using a commercial array.

ILT2-WT and *ILT2*-MT transfect-Jurkat cell line treated with PV were lysed, incubated with the R&D Systems Human Phospho-Immunoreceptor Antibody Array, washed, and incubated with a phosphotyrosine-specific antibody, and results were visualized by chemiluminescence. A representative blot of *ILT2* and *SHP-1* is shown in red and blue blocks, respectively(A). Representative dot blot graphs showing phosphorylation level (B)

After PV treatment induced substantial tyrosine phosphorylation of *ILT2*, we found phosphorylation level of *ILT2* and *SHP-1* decreased *ILT2*-MT transfect-Jurkat cells at 10 mins when compare with *ILT2*-WT transfect-Jurkat cells. These suggest that these *ILT2* mutations are likely to cause the disease by a loss-of-function mechanism.

Chapter V

Discussion

Autoimmune diseases (AD), triggered by genetic and environmental factors that failed the immune system to develop tolerance toward self-antigens, are characterized by immune responses against self-tissues. Until now, the cause of the autoimmune disease is not well understood. Currently, it is estimated that only 15% of the genetic factor contributing to autoimmune disease. However, there are several genes that still possibly cause this disease. Although, there are many studies identify known gene which related to AD. But only a few percent of the patient carrying a genetic variant of the known gene linked to this disease.

Here, we identified a three-generation family with 9 members suffering from one of the three autoimmune diseases – Grave's disease, Hashimoto thyroiditis, or SLE. In this study, the WGL and WES determine genetic variants in familial with autoimmune disease. WGL revealed a locus on chromosome 19 with the maximum LOD score of 2.04. WES identified a heterozygous missense mutation, c.479G>A (p. G160E) *ILT2*, located within the linked region, in all affected members. Moreover, we also identified this novel variant in three unaffected individuals who did not have the disease. The mutation segregated with the disease phenotype in the family with 75% penetrance. Ideally, the causal variant is present only in affected individuals, but unaffected carriers can occur because of incomplete penetrance or late-onset autoimmune disease (38).

Autoimmune diseases result from a loss of immune tolerance; it has been described in different animal models that the defective function or absence of regulatory receptors expressed by immune cells and those with inhibitory motifs (ITIMs), including ILT molecules, is associated with autoimmune diseases (39). The previous study shows that different receptors have been described to negatively regulate immune responses by bearing inhibitory motifs (ITIMs) in their cytoplasmic tails, including ILT2, implicated in innate and adaptive immunity (40). Alterations in the function of these regulatory mechanisms have been associated with the development of inflammatory disorders, autoimmune diseases, and lymphoid malignancies (41). In this regard, previous studies have suggested that *ILT2* receptors play an important role in the regulation of the immune response in different conditions, including systemic lupus erythematosus (17), rheumatoid arthritis (35), and autoimmune thyroiditis (4).

Immunoglobulin-like transcript receptor 2 (*ILT2* /LILRB1/CD85j) is an inhibitory receptor broadly expressed on leucocytes and recognizes human antigen G (HLA-G). *ILT2* is expressed on various proportions of NK cells, CD8+ lymphocytes, and CD4+ lymphocytes, as well as B lymphocytes, monocytes, and dendritic cells. The identified c.479G>A variant in the *ILT2* gene changes a nucleotide at position 479 from G to A (c.479G>A), leading to amino acid changing from glycine to glutamic acid at position 160 (p.G160E). The mutation is located on the immunoglobulin-like domain (Ig-like C2-type 2), an important region for binding with a major histocompatibility complex, class I. The p. G160E was predicted to be deleterious and

located in the D1D2 domain playing an essential role in binding HLA I and regulating inhibitory signal (42, 43). The pathogenicity of the *ILT2* variant, p. G160E, was then investigated.

In this study, Flow cytometric immunophenotyping of PBMC from autoimmune disease patients with HT, GD, and SLE showed no difference in the percent of CD4+ lymphocytes, CD8+ lymphocytes, CD56+, CD19+, monocyte, myeloid dendritic cell (mDCs), plasmacytoid dendritic cell (pDCs) compared with controls.

ILT2 can down-regulate T and B cell activation and proliferation (44). In this study, we hypothesized the percentage of T and B cells of patients should increase due to loss-of-function mutation in ILT2, leading to T and B cell overactivity. However, the present study showed no significant differences in the percentage of CD4+ and CD8+ cells compared with control. It could be explained by the lower number of patients in this study. The limitation of this study is it was not able to know these cells are autoreactive cells. In contrast to B cell, the present result shows that percentage of B cell is higher than controls. Therefore, it might be speculated that *the ILT2* variant result in defective function may relate to hyperactivity B cell.

For pDCs, the previous study found significantly low levels of pDCs in the peripheral blood from AITD patients; however, Monsiváis-Urenda et al found that SLE patients showed significantly higher levels of circulating pDCs compared to healthy control. In contrast, the levels of mDCs were similar in HT, GD,SLE patients, and controls. It might be speculated that the level of DCs in the PBMC of patients are variable, are stratified by age, and that disease activity and other undetermined factors regulate them (25, 45) .

The present study also showed no significant differences in expression of the inhibitory receptors *ILT2* in CD4+, CD8+, CD19+, CD56+, and dendritic cells compared with control. This could be explained that this variant is not located within the promoter region that can affect the expression level.

Notably, many studies previously show controversial expression levels, and they found a variable level of expression of *ILT2* by lymphocytes from both SLE patients and healthy controls. Therefore, they suggest considering that it would be of interest to determine the possible presence of SNPs in the *ILT2* gene that would be associated with a low level of expression or cause an enhanced synthesis of the protein, and their possible association with SLE and AITD, respectively.

Plasmablasts producing pathogenic autoantibodies in patients with systemic autoimmune diseases (46); the present result shows that percentage of plasmablast is higher than controls. According to the study of Inui et al. in 2015, isoforms of *ILT/LILR*, whose murine homolog is Paired Ig-like receptor (Pir)B (47), a negative regulator of auto-antibody production, a close homolog of murine PirB. It might be speculated that *the ILT2* variant result in defective function may relate to hyperactivity B cell. And increase the production of autoantibody, which might be linked to the development of autoimmune disease.

Furthermore, this study is the first time to explore *ILT2*+ on the Breg, transitional B cell, plasmablast in autoimmune disease. However, we cannot find any difference in percentage and expression of Breg *ILT2*+ cell. Thus, our findings are preliminary and need more extensive

studies on the expression *of ILT2* and Breg phenotypes to shed light on the behavior of these cells in autoimmune diseases.

Several publications have reported a strong correlation between the LAP+/GARP+ phenotype and suppressive function Treg cell and show that CD4+GARP+/-LAP+ Tregs make IL-10 immunosuppressive cytokine but not IFN-γ effector cytokine (48). In addition, the previous study shows the decreased number of Treg is related to the pathogenesis of autoimmune. However, our findings cannot show any difference in percentage Treg and with Treg *ILT2*+ cell. Therefore, it is possible to speculate that the reported variations of Treg status among autoimmune patients are attributable to inconsistent Treg identification; different markers are employed (46).

Interestingly, we found the percentage of GARP+IL2+ Treg significantly decrease in affected cases. GARP is a cell surface molecule of Tregs that maintains their regulatory function and homeostasis, suggesting that dysregulation of ILT2 may involve the induction of Treg cells. Furthermore, the surface expression of *ILT2* is reduced on *LAP+ILT2+* Treg (mean of mean fluorescence intensity (MFI): 366 ± 105 vs. 685 ± 251, P<0.005), *GARP+ILT2+*Treg (MFI: 283± 38 vs. 424 ± 104 P<0.005), and *LAP+GARP+ILT2+* Treg from patients compared with controls, *ILT2* expression (MFI: 417± 124 vs. 653 ± 368 P<0.005)

· Moreover, we use HP-F1, the mimic ligand of *ILT2*, to activate the receptor. Nevertheless, we found the surface expression of *ILT2* is reduced on *LAP+ILT2+* from patients compared with controls (mean of mean fluorescence intensity (MFI): 328 ± 94 vs. 535 ± 113, P<0.005).

Previously reveal that HLA-G on DCs interacts with *ILT2* on T cells, which drives differentiation of T cells into IL-10 producing Type 1 T regulatory. They might be variant of ILT2 maybe exert a defective regulatory function contributing to the diminished activity of Treg lymphocytes previously described in this condition (28, 49, 50)

Due to the small number of patients in this study, we did not see the percentage and expression of *ILT2* of any patient's immune cell different from controls.

In our present study, the expressions of IL-2, IL-6, IL-10, and IL-12 were showed no appearance of significant difference in PBMCs of 8 affected individuals with *ILT2* mutation patients and 3 unaffected individuals with *ILT2* mutation than in PBMCs from 2 unaffected controls. Similarly, the previous study PBMC from AITD patients showed the synthesis of IL2 on *ILT2* engagement was not significantly different in the HT, GD, and control groups. On the contrary, they found a diminished synthesis of interleukin 10 on ILT2 engagement. Thus, they suggest that the regulatory function of *ILT2* is also mediated by a strong induction of synthesis of the anti-inflammatory cytokine IL10.

Interestingly, in moDcs, Urena et al. found *ILT2* engagement tended to enhance the release of IL6 in moDCs from both controls and patients but not significant. Their data suggest that *ILT2* does not seem to have a relevant role in regulating the synthesis of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and IL12 release by DCs from SLE patients. All these data suggest that *ILT2* has a complex role in the synthesis and release of cytokines by isolated DCs and during the interaction of these cells with T lymphocytes. Thus, the regulatory function of

ILT2 on the synthesis of cytokines in autoimmune patients remains an interesting point to be defined.

Since we did not find any different phenotype of a distinct immune cell population from flow cytometry, we decided to use single-cell RNA sequencing (scRNA-seq) to define, characterize disease-related immune cell subset and identify the transcriptional signatures of these immune cells that may be correlated with pathogenicity PBMC of 3 patients and control. Interestingly, scRNA-sequencing reveals monocyte is the increased percentage found in patients.

Monocytes possess broad immuno-modulatory, inflammatory, and tissue-repairing capabilities and actively participate in developing many autoimmune diseases. The previous study Analysis by flow cytometry revealed that deletion of *LILRB1* from macrophages *in vitro* resulted in a significantly greater abundance of cells with an M1-like surface profile than that among macrophages treated with an off-target control sgRNA (51). Notably, M1 monocyte subsets, which were previously observed to play a role in inflammation and autoimmune diseases, in patients were not different from the control. Although we do not have a commendable explanation for these contrasting results, it may be from the effect of variant is may sufficient to promote monocyte to M2.

Next, the pathomechanism study of p. G160E showed that phosphorylation of *ILT2* and *SHP-1* decrease when compared with wild-type. These indicate that it possesses a loss-of-function mechanism. The present research supports the function of *ILT2* and T cell activation.

The further experiment involving an observed reduction in tyrosine phosphorylation of downstream pathway is required to evaluate the effect of the mutant variant in T cell activation of patients with familial autoimmune disease. This is the first time to implicate *ILT2* as a possible new disease gene for autoimmunity.

REFERENCES

1. Sinha AA, Lopez MT, McDevitt HO. Autoimmune diseases: the failure of self tolerance. Science. 1990;248(4961):1380-8.

2. Stassi G, De Maria R. Autoimmune thyroid disease: new models of cell death in autoimmunity. Nat Rev Immunol. 2002;2(3):195-204.

3. Mills JA. Systemic lupus erythematosus. N Engl J Med. 1994;330(26):1871-9.

4. Doniz-Padilla L, Paniagua AE, Sandoval-Correa P, Monsivais-Urenda A, Leskela S, Marazuela M, et al. Analysis of expression and function of the inhibitory receptor ILT2 in lymphocytes from patients with autoimmune thyroid disease. Eur J Endocrinol. 2011;165(1):129-36.

5. See P, Lum J, Chen J, Ginhoux F. A Single-Cell Sequencing Guide for Immunologists. Front Immunol. 2018;9:2425.

6. Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. Nat Rev Immunol. 2018;18(1):35-45.

7. Monsivais-Urenda A, Nino-Moreno P, Abud-Mendoza C, Baranda L, Layseca-Espinosa E, Lopez-Botet M, et al. Analysis of expression and function of the inhibitory receptor ILT2 (CD85j/LILRB1/LIR-1) in peripheral blood mononuclear cells from patients with systemic lupus erythematosus (SLE). J Autoimmun. 2007;29(2-3):97-105.

8. Dietrich J, Cella M, Colonna M. Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1) inhibits TCR signaling and actin cytoskeleton reorganization. J Immunol. 2001;166(4):2514-21.

9. Caturegli P, Kimura H, Rocchi R, Rose NR. Autoimmune thyroid diseases. Curr Opin Rheumatol. 2007;19(1):44-8.

10. Devendra D, Eisenbarth GS. 17. Immunologic endocrine disorders. J Allergy Clin Immunol. 2003;111(2 Suppl):S624-36.

11. Weetman AP. Autoimmune thyroid disease: propagation and progression. Eur J Endocrinol. 2003;148(1):1-9.

12. Ban Y, Tomer Y. Susceptibility genes in thyroid autoimmunity. Clin Dev Immunol. 2005;12(1):47-58.

13. Ban Y, Tozaki T, Taniyama M, Tomita M, Ban Y. Association of a CTLA-4 3' untranslated region (CT60) single nucleotide polymorphism with autoimmune thyroid disease in the Japanese population. Autoimmunity. 2005;38(2):151-3.

14. Manson JJ, Rahman A. Systemic lupus erythematosus. Orphanet J Rare Dis. 2006;1:6.

15. Deng Y, Tsao BP. Genetic susceptibility to systemic lupus erythematosus in the genomic era. Nat Rev Rheumatol. 2010;6(12):683-92.

16. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. Nat Rev Immunol. 2001;1(3):220-8.

17. Brown D, Trowsdale J, Allen R. The LILR family: modulators of innate and adaptive immune pathways in health and disease. Tissue Antigens. 2004;64(3):215-25.

18. Chen J, Shen B, Jiang Y, Jun L, Zhu M, Chen B, et al. Analysis of immunoglobulin-like transcripts (ILTs) in lymphocytes with sHLA-G and IL10 from SLE patients. Clin Exp Med. 2013;13(2):135-42.

19. Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med. 1997;186(11):1809-18.

20. Kuroki K, Furukawa A, Maenaka K. Molecular recognition of paired receptors in the immune system. Front Microbiol. 2012;3:429.

21. Zhang F, Zheng J, Kang X, Deng M, Lu Z, Kim J, et al. Inhibitory leukocyte immunoglobulin-like receptors in cancer development. Sci China Life Sci. 2015;58(12):1216-25.

22. Sayos J, Martinez-Barriocanal A, Kitzig F, Bellon T, Lopez-Botet M. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. Biochem Biophys Res Commun. 2004;324(2):640-7.

23. Rouas-Freiss N, Moreau P, LeMaoult J, Carosella ED. The dual role of HLA-G in cancer. J Immunol Res. 2014;2014:359748.

24. Lin A, Yan WH. Heterogeneity of HLA-G Expression in Cancers: Facing the Challenges. Front Immunol. 2018;9:2164.

25. Monsivais-Urenda A, Gomez-Martin D, Santana-de-Anda K, Cruz-Martinez J, Alcocer-Varela J, Gonzalez-Amaro R. Defective expression and function of the ILT2/CD85j regulatory

receptor in dendritic cells from patients with systemic lupus erythematosus. Hum Immunol. 2013;74(9):1088-96.

26. Zhang X, Olsen N, Zheng SG. The progress and prospect of regulatory T cells in autoimmune diseases. J Autoimmun. 2020;111:102461.

27. Xu X, Zhou Y, Wei H. Roles of HLA-G in the Maternal-Fetal Immune Microenvironment. Front Immunol. 2020;11:592010.

28. Marazuela M, Garcia-Lopez MA, Figueroa-Vega N, de la Fuente H, Alvarado-Sanchez B, Monsivais-Urenda A, et al. Regulatory T cells in human autoimmune thyroid disease. J Clin Endocrinol Metab. 2006;91(9):3639-46.

29. Abd Al Samid M, Chaudhary B, Khaled YS, Ammori BJ, Elkord E. Combining FoxP3 and Helios with GARP/LAP markers can identify expanded Treg subsets in cancer patients. Oncotarget. 2016;7(12):14083-94.

30. Rydzewska M, Jaromin M, Pasierowska IE, Stozek K, Bossowski A. Role of the T and B lymphocytes in pathogenesis of autoimmune thyroid diseases. Thyroid Res. 2018;11:2.

31. Stubbington MJT, Rozenblatt-Rosen O, Regev A, Teichmann SA. Single-cell transcriptomics to explore the immune system in health and disease. Science. 2017;358(6359):58-63.

32. Fujio K, Takeshima Y, Nakano M, Iwasaki Y. Review: transcriptome and trans-omics analysis of systemic lupus erythematosus. Inflamm Regen. 2020;40:11.

33. Arazi A, Rao DA, Berthier CC, Davidson A, Liu Y, Hoover PJ, et al. The immune cell landscape in kidneys of patients with lupus nephritis. Nat Immunol. 2019;20(7):902-14.

34. Hemwong N, Phokaew C, Srichomthong C, Tongkobpetch S, Srilanchakon K, Supornsilchai V, et al. A patient with combined pituitary hormone deficiency and osteogenesis imperfecta associated with mutations in LHX4 and COL1A2. J Adv Res. 2020;21:121-7.

35. Kuroki K, Tsuchiya N, Shiroishi M, Rasubala L, Yamashita Y, Matsuta K, et al. Extensive polymorphisms of LILRB1 (ILT2, LIR1) and their association with HLA-DRB1 shared epitope negative rheumatoid arthritis. Hum Mol Genet. 2005;14(16):2469-80.

36. Hu Y, Hu Y, Xiao Y, Wen F, Zhang S, Liang D, et al. Genetic landscape and autoimmunity of monocytes in developing Vogt-Koyanagi-Harada disease. Proc Natl Acad Sci U S A. 2020;117(41):25712-21.

37. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. Nat Methods. 2015;12(5):453-7.

38. Bias WB, Reveille JD, Beaty TH, Meyers DA, Arnett FC. Evidence that autoimmunity in man is a Mendelian dominant trait. Am J Hum Genet. 1986;39(5):584-602.

39. Anderson KJ, Allen RL. Regulation of T-cell immunity by leucocyte immunoglobulin-like receptors: innate immune receptors for self on antigen-presenting cells. Immunology. 2009;127(1):8-17.

40. Long EO. Regulation of immune responses through inhibitory receptors. Annu Rev Immunol. 1999;17:875-904.

41. Veillette A, Latour S, Davidson D. Negative regulation of immunoreceptor signaling. Annu Rev Immunol. 2002;20:669-707.

42. Chapman TL, Heikema AP, West AP, Jr., Bjorkman PJ. Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2). Immunity. 2000;13(5):727-36.

43. Lichterfeld M, Yu XG. The emerging role of leukocyte immunoglobulin-like receptors (LILRs) in HIV-1 infection. J Leukoc Biol. 2012;91(1):27-33.

44. Villa-Alvarez M, Lorenzo-Herrero S, Gonzalez-Rodriguez AP, Lopez-Soto A, Payer AR, Gonzalez-Garcia E, et al. Ig-like transcript 2 (ILT2) suppresses T cell function in chronic lymphocytic leukemia. Oncoimmunology. 2017;6(10):e1353856.

45. Leskela S, Rodriguez-Munoz A, de la Fuente H, Figueroa-Vega N, Bonay P, Martin P, et al. Plasmacytoid dendritic cells in patients with autoimmune thyroid disease. J Clin Endocrinol Metab. 2013;98(7):2822-33.

46. Zhang SX, Ma XW, Li YF, Lai NL, Huang ZH, Fan K, et al. The Proportion of Regulatory T Cells in Patients with Systemic Lupus Erythematosus: A Meta-Analysis. J Immunol Res. 2018;2018:7103219.

47. Inui M, Hirota S, Hirano K, Fujii H, Sugahara-Tobinai A, Ishii T, et al. Human CD43+ B cells are closely related not only to memory B cells phenotypically but also to plasmablasts developmentally in healthy individuals. Int Immunol. 2015;27(7):345-55.

48. Elkord E, Abd Al Samid M, Chaudhary B. Helios, and not FoxP3, is the marker of activated Tregs expressing GARP/LAP. Oncotarget. 2015;6(24):20026-36.

49. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. Blood. 2010;116(6):935-44.

50. Mao C, Wang S, Xiao Y, Xu J, Jiang Q, Jin M, et al. Impairment of regulatory capacity of CD4+CD25+ regulatory T cells mediated by dendritic cell polarization and hyperthyroidism in Graves' disease. J Immunol. 2011;186(8):4734-43.

51. Barkal AA, Weiskopf K, Kao KS, Gordon SR, Rosental B, Yiu YY, et al. Engagement of MHC class I by the inhibitory receptor LILRB1 suppresses macrophages and is a target of cancer immunotherapy. Nat Immunol. 2018;19(1):76-84.

APPENDIX

Table 7 Raw quantitative qPCR cycle threshold for *ILT2* gene and housekeeping gene

Sample/Ct	*ILT2*	*ACTB*
SES001	24.81099892	22.84152412
SES001	24.79642487	22.79307175
SES001	24.77489471	22.8177681
PCP002	25.15083694	23.21062851
PCP002	24.73522186	23.23541641
PCP002	24.75885773	23.32117653
MTT003	25.4679184	21.10770798
MTT003	25.40640259	21.17130089
MTT003	25.30670166	21.15236473
YES004	25.02068901	20.87182617
YES004	24.97981262	20.81151962
YES004	24.97380066	20.80985069
SES005	24.09654808	23.68130112
SES005	24.04851341	23.69371223
SES005	24.03569603	23.85043716
CCP006	24.87116432	21.10860062
CCP006	24.79323196	21.0716114
CCP006	24.80326843	21.10997581
NSJ007	25.23982239	23.95455551
NSJ007	25.14381218	23.8064518
NSJ007	25.14233017	23.90485764

Table 8 Raw quantitative qPCR cycle threshold for *ILT2* gene and housekeeping gene *(continue)*

Sample/Ct	*ILT2*	*ACTB*
SLW008	25.48784828	21.61462975
SLW008	25.21064186	21.62091064
SLW008	25.11768341	21.64335632
PCP009	24.61529732	22.96648979
PCP009	24.74802017	23.3341217
PCP009	24.74815941	22.90432167
PCP010	24.93735313	23.51348686
PCP010	24.84348488	22.91254044
PCP010	24.92094612	23.17018127
SCP011	24.37787628	21.55729294
SCP011	24.68408585	21.65568352
SCP011	24.4712677	21.59203148
PCP012	25.53250694	21.01187515
PCP012	25.60931015	21.07184982
PCP012	26.31760406	21.15727806
PCP014	24.19166946	22.73944092
PCP014	24.36693573	22.28008842
PCP014	24.74189186	21.71560478

Table 9 Raw quantitative qPCR cycle threshold for IL2, IL6, IL10 and IL12 gene and housekeeping gene

Sample/Ct	*IL2*	*IL6*	*IL10*	*IL12B*	*ACTB*
SES001	33.96820068	28.41571999	30.95812035	32.51758957	22.97907
SES001	33.93734741	28.43600464	30.86548805	Undetermined	22.99155
SES001	33.94367599	28.39863396	30.93278885	34.78123474	22.84934
PCP002	34.17018127	30.05539513	32.45962906	34.09355545	23.32755
PCP002	34.52031326	30.08147812	32.37880707	33.50653458	23.32639
PCP002	33.96354294	30.00709724	32.0663681	33.76539993	23.49748
MTT003	35.02183533	31.12457085	30.24125481	32.30106735	21.20571
MTT003	34.97624207	31.08854675	30.35802078	33.71735764	21.20812
MTT003	35.55406952	31.01411438	30.1427269	33.22208786	21.30986
YES004	34.44910049	29.56454659	29.73748207	Undetermined	20.76237
YES004	34.58399582	29.42879105	29.84578133	32.3920784	20.85283
YES004	34.30091858	29.51219559	29.62441826	33.15877914	20.85468
SES005	36.5368042	29.11447525	33.51157761	34.25808334	23.74657
SES005	36.50169754	28.9853878	33.59558868	36.08484268	23.78854
SES005	36.96553421	28.97213745	33.8559761	35.51660156	23.64026
CCP006	35.15721512	28.54626083	30.01682472	31.82527351	21.29687
CCP006	34.96701431	28.49605179	29.98294258	32.60434723	21.0396
CCP006	34.73144913	28.5677681	29.85538673	32.46427155	21.2947
NSJ007	Undetermined	28.52039909	36.19831848	35.29471207	24.12611
NSJ007	Undetermined	28.76455879	35.81100464	35.18296051	23.99569
NSJ007	Undetermined	28.6391449	37.02189636	Undetermined	24.09546

Table 10 Raw quantitative qPCR cycle threshold for IL2, IL6, IL10 and IL12 gene and housekeeping gene *(continued)*

Sample/Ct	*IL2*	*IL6*	*IL10*	*IL12B*	*ACTB*
SLW008	Undetermined	29.29174995	30.63734245	36.71513748	21.86122
SLW008	Undetermined	27.41168404	30.41677475	33.66236496	21.89276
SLW008	Undetermined	27.29768562	30.5359726	38	21.77699
PCP009	35.42067719	31.02435112	32.40021896	36.87699509	23.11603
PCP009	35.49444199	30.96964264	32.64118576	35.08045959	23.0743
PCP009	35.83180618	31.08144379	32.65320587	33.80422592	23.10949
PCP010	34.70186234	29.82219887	32.08782959	Undetermined	23.50572
PCP010	35.57395172	29.71213531	31.67526054	33.05997849	23.57764
PCP010	35.31621552	29.93340874	31.89484978	Undetermined	23.55907
SCP011	35.84363937	28.21376991	30.07806587	31.92170334	21.76818
SCP011	35.73564529	28.28541565	30.29653931	32.84022141	21.98118
SCP011	35.77408981	28.300354	30.2029171	Undetermined	21.86242
PCP012	34.79568481	35.47515488	30.69590759	30.44030571	21.49976
PCP012	34.35548019	35.07485199	30.80278015	31.77414322	21.50795
PCP012	34.53660202	35.98540497	30.47979927	29.76989746	21.47717
PCP014	34.75101471	28.81303024	31.59274292	32.41577911	22.9401
PCP014	34.61928177	28.19552994	31.80295753	34.19787598	22.92075
PCP014	34.72221756	28.30047035	31.86515999	34.81784439	22.9069

VITA

NAME	Thivaratana Sinthuwiwat
DATE OF BIRTH	6 Sep 1980
PLACE OF BIRTH	Bangkok
HOME ADDRESS	20/83 lumpini place Ramindra-laksri Ramindra Rd. Bangkhen BKK Thailand