CHARACTERIZATION OF MAIT TCR REPERTOIRE OF GASTROINTESTINAL TRACT AND PERIPHERAL BLOOD MAIT CELLS

Miss Thidarat Kongkaew

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Microbiology
Medical Microbiology,Interdisciplinary Program
GRADUATE SCHOOL
Chulalongkorn University
Academic Year 2019

การศึกษาคุณลักษณะของ MAIT TCR repertoire บริเวณทางเดินอาหารและเลือด

น.ส.ธิดารัตน์ กองแก้ว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ สหสาขาวิชาจุลชีววิทยาทางการแพทย์
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

Thesis Title	CHARACTERIZATION OF MAIT TCR REPERTOIRE OF GASTROINTESTINAL TRACT AND PERIPHERAL BLOOD MAIT CELLS
By	Miss Thidarat Kongkaew
Field of Study	Medical Microbiology
Thesis Advisor	Rangsima Reantragoon, Ph.D.

Accepted by the GRADUATE SCHOOL, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the GRADUATE SCHOOL
(Associate Professor Thumnoon Nhujak, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Professor Nattiya Hirankarn, Ph.D.)

..... Thesis Advisor
(Rangsima Reantragoon, Ph.D.)

..... External Examiner
(Assistant Professor Ponpan Matangkasombut Choopong, Ph.D.)

ธิดารัตน์ กองแก้ว : การศึกษาคุณลักษณะของ MAIT TCR repertoire บริเวณทางเดินอาหารและเลือด. (CHARACTERIZATION OF MAIT TCR REPERTOIRE OF GASTROINTESTINAL TRACT AND PERIPHERAL BLOOD MAIT CELLS) อ.ที่ปรึกษาหลัก : พญ. ดร.รังสิมา เหรียญตระกูล

เมตต์เซลล์เป็นทีเซลล์ชนิดหนึ่งที่สามารถตอบสนองต่อการติดเชื้อได้อย่างรวดเร็ว เมตต์เซลล์เป็นแอลฟ่าเบต้าทีเซลล์ที่พบสายแอลฟ่าเป็น Vα 7.2 (TRAV1-2) เป็นส่วนใหญ่ และมักจะจับคู่กับ TRAJ33, TRAJ20 หรือ TRAJ12 ส่วนสายเบต้า มีความหลาหลายมากกว่าสายแอลฟ่า แต่จะพบ TRBV20-1, TRBV6-1 หรือ TRBV6-4 มากกว่าชนิดอื่นๆ ก่อนหน้านี้มีผู้วิจัยได้ศึกษาความหลากหลายของเมตต์ทีเซลล์รีเซปเตอร์ในเลือด อย่างไรก็ตามยังไม่มีรายงานถึงการศึกษาเมตต์เซลล์ในทางเดินอาหาร ดังนั้นในการศึกษานี้ผู้วิจัยต้องการศึกษาเมตต์ทีเซลล์รีเซปเตอร์ที่แยกได้จากดูโอดีนัม ไอเลียม โคลอน และPBMC ในการศึกษานี้ผู้วิจัยได้ศึกษาในผู้ป่วย 2 รายที่ได้รับการส่องกล้องเพื่อคัดกรองโรค โดยได้เก็บตัวอย่างเลือดและชิ้นเนื้อจากทางเดินอาหาร จากนั้นทำการแยก PBMCs จากเลือด และ LPLs จากชิ้นเนื้อ เนื่องจากจำนวนเซลล์ที่แยกได้จากชิ้นเนื้อมีจำนวนน้อยและไม่เพียงพอจึงต้องทำการเพิ่มจำนวน ซึ่งพบว่าเมตต์เซลล์สามารถเพิ่มจำนวนในสภาพแวดล้อมที่สร้างขึ้นได้ และเมื่อศึกษาความหลากหลายของทีเซลล์รีเซปเตอร์ พบ TRAJ33, TRAJ20 และ TRAJ12 มากที่สุด ซึ่งมี Tyr95 เป็นตัวสำคัญในการสื่อสารกับแอนติเจน และยังพบ TRAJ30 และ TRAJ39 ซึ่งมี Arg95 และ Asn95 เป็นตัวสื่อสารกับแอนติเจนได้เช่นกัน นอกจากนี้พบว่า ทุกบริเวณมีการเลือกใช้ยีน TR20-1 และ TRBV6-1 มากที่สุด และการเลือกใช้ TRBV ในแต่บริเวณมีทั้งความเหมือนและความต่างกัน โดยเฉพาะในบริเวณไอเลียมมีความหลากหลายมากที่สุด จากกงานวิจัยผู้วิจัยสรุปว่า ของเมตต์ทีเซลล์รีเซปเตอร์ที่บริเวณเยื่อบุผิวมีความหลากหลายมากกว่าบริเวณเลือด และผลการทดลองที่ได้จากความหลากหลายของเมตต์ที่เซลล์รีเซปเตอร์คาดว่าความหลากหลายของเมตต์ทีเซลล์รีเซปเตอร์มีความเกี่ยวข้องกับการคัดเลือกของแบคทีเรีย อย่างไรก็ตามการศึกษาเพิ่มเติมเกี่ยวกับแบคทีเรียที่สามารถสังเคราะห์วิตามินบีเมตาโบไลท์ จะช่วยให้ทราบถึงการคัดเลือกของเมตต์ทีเซลล์รีเซปเตอร์ได้ดีขึ้น

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5987148920 : MAJOR MEDICAL MICROBIOLOGY
KEYWORD: GASTROINTESTINAL TRACT, PERIPHERAL BLOOD, MUCOSAL IMMUNITY, MAIT CELLS, TCR REPERTOIRE

Thidarat Kongkaew : CHARACTERIZATION OF MAIT TCR REPERTOIRE OF GASTROINTESTINAL TRACT AND PERIPHERAL BLOOD MAIT CELLS. Advisor: Rangsima Reantragoon, Ph.D.

Mucosal-associated invariant T (MAIT) cells are an innate-like T cell that rapidly respond to infection. MAIT cells are αβ T cells that possess a Vα7.2 (TRAV1-2) TCR with mostly pairing to TRAJ33, TRAJ20 or TRAJ12 and predominantly TRBV20-1, TRBV6-1 and TRBV6-4. MAIT T cell receptors (TCR) repertoire have been previously studied in peripheral blood. However, data on MAIT TCR repertoire in gastrointestinal tract are lacking. We studied 2 individuals who are underwent esophagogastroduodenoscopy (EGD) and colonoscopy. Blood and biopsies were obtained. PBMCs were isolated from blood and LPLs were isolated from biopsies. Due to the low number of MAIT cells in each gastrointestinal tract site and being insufficient for experiments, we proliferated MAIT cells to increase MAIT cell population. Our results show that MAIT cells can proliferate in vitro. We mostly found TRAJ33 followed by TRAJ20, TRAJ12 that contained Tyr95, which necessary for interaction with antigens and TRAJ30 and TRAJ36, which contained Arg95 and Asn95 that also interact with antigens. Moreover, most TRBV gene usage in every site were TRBV6-4 and TRBV20-1. There were overlapping and non-overlapping TRBV gene usage in each site. The TRBV in ileum of both patients was the most diverse. We may concluded that MAIT TCR repertoire in mucosal was more diverse than peripheral and the result of MAIT TCR repertoire may concluded that MAIT TCR repertoire are associate with bacterial selection. However, the study of vitamin B metabolite synthesis bacteria may be help us to more understand MAIT TCR selection.

Field of Study: Medical Microbiology Student's Signature .....
Academic Year: 2019 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my gratitude to every person who was involved in the complettion of thesis. I would like to thank Assist. Prof. Rangsima Reantragoon, M.D., Ph.D, my advisor at the Division of Immunology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, who kindly to teach, guide, support, and encourage me throughout the course of study.

I would like to thanks the staffs at the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, who helped on my work. I would like to thank my senior, friend classmate and staffs at Medical Microbiology, Interdisciplinary Program, Graduate School, Chulalongkorn University. Special thanks to the people in Center of Excellence Immunology and Immune-Mediated Diseases, Faculty of Medicine, Chulalongkorn University for all their help.

Finally, I would like to thank the scholarship from Graduate School, Chulalongkorn University to commemorate The Celebrations on the Auspicious Occasion of Her Roral Highness Princess Maha Chakri Sirindhon's 5th Cycle (60th) Birthday.

Thidarat Kongkaew

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF ABBREVIATION

AMP	=	Antimicrobial peptides
Blimp-1	=	B cell lymphocyte-induced maturation protein-1
C/EBPδ	=	CCAAT/enhancer-binding protein delta
CCL2	=	C-C motif chemokine receptor 2
CCL5	=	C-C motif chemokine receptor 5
CCL7	=	C-C motif chemokine receptor 7
CCL9	=	C-C motif chemokine receptor 9
CCL10	=	C-C motif chemokine receptor 10
CD1d	=	Cluster of differentiation 1d
CD3	=	Cluster of differentiation 3
CD4	=	Cluster of differentiation 4
CD8	=	Cluster of differentiation 8
CD8$\alpha\alpha$	=	Cluster of differentiation 8 alpha alpha
CD8$\alpha\beta$	=	Cluster of differentiation 8 alpha beta
CD11c	=	Cluster of differentiation 11c
CD103	=	Cluster of differentiation 103
CD161	=	Cluster of differentiation 161
CDR	=	complementarity-determining region
CXCR6	=	C-X-C motif chemokine receptor 6
DC	=	Dendritic cell
DMSO	=	Dimethyl sulfoxide
EOMES	=	Eomesodermin
EGD	=	Esophagogastroduodenoscopy
FCS	=	Fetal calf serum
GI Tract	=	Gastrointestinal tract
IBD	=	Inflammatory bowel diseases
IELs	=	Intra-epithelial lymphocytes
IFN-αR	=	Interferon-alpha receptor
IFN-γ	=	Interferon-gamma

sIgA	=	secretory Immunoglobulin A
IgE	=	Immunoglobulin E
Jα12	=	Joining alpha 12
Jα20	=	Joining alpha 20
Jα33	=	Joining alpha 33
IL-1β	=	Interleukin 1β
IL-2	=	Interleukin 2
IL-2Rβ	=	Interleukin 2 receptor beta
IL-4	=	Interleukin 4
IL-5	=	Interleukin 5
IL-6	=	Interleukin 6
IL-7Rα	=	Interleukin 7 receptor alpha
IL-10	=	Interleukin 10
IL-12	=	Interleukin 1
IL-13	=	Interleukin 13
IL-17	=	Interleukin 17
IL-18Rα	=	Interleukin 18 receptor alpha
IL-21	=	Interleukin 21
IL-22	=	Interleukin 22
IL-23	=	Interleukin 23
IL-23R	=	Interleukin 23 receptor
IL-25	=	Interleukin 25
IL-33	=	Interleukin 33
ILC	=	Innate lymphoid cell
iNKT cell	=	Invariant natural killer T cell
LPLs	=	Lamina propria lymphocytes
M cell	=	Microfold cell
MAIT cell	=	Mucosal-associated invariant T cell
MR1	=	Majer histrocompattibility complex-related molecule 1

NKG2D	=	Natural killer group 2D
NK cell	=	Natural killer cell
NKT cell	=	Natural killer T cell
PBMC	=	Peripheral blood mononuclear cell
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
PLZF	=	Promyelocytic leukemia zinc finger
RA	=	Rhumatoid arthritis
RegIIIγ	=	Regenerating islet-derived protein 3 gamma
RORγt	=	Retinoic acid receptor-related orphan receptor gamma t
RPM	=	round per minite
STAT3	=	Signal transducer and activator of transcription 3
TAE buffer	=	Tris-acetate-EDTA buffer
T-bet	=	T-box expression in T cell
Tfh	=	Follicular helper T cells
TGF-β	=	Transforming growth factor beta
Th1 cell	=	T helper 1 cell
Th2 cell	=	T helper 2 cell
Th17 cell	=	T helper 17 cell
Th22 cell	=	T helper 22 cell
TNF-α	=	Tumor necrosis factor-alpha
TCR	=	T cell receptor
TCRα	=	T cell receptor alpha
TCRβ	=	T cell receptor beta
TRAV1-2	=	T cell receptor alpha variable 1-2
TRAJ12	=	T cell receptor joining 12
TRAJ20	=	T cell receptor joining 20
TRAJ30	=	T cell receptor joining 30
TRAJ33	=	T cell receptor joining 33

TRAJ36	=	T cell receptor joining 36
TRBV2	=	T cell receptor beta 2
TRBV3-1	=	T cell receptor beta 3-1
TRBV3-2	=	T cell receptor beta 3-2
TRBV4-2	=	T cell receptor beta 4-2
TRBV5-8	=	T cell receptor beta 5-8
TRBV6-1	=	T cell receptor beta 6-1
TRBV6-4	=	T cell receptor beta 6-4
TRBV7-2	=	T cell receptor beta 7-2
TRBV7-8	=	T cell receptor beta 7-9
TRBV9	=	T cell receptor beta 9
TRBV10-1	=	T cell receptor beta 10-1
TRBV11-1	=	T cell receptor beta 11-1
TRBV12-3	=	T cell receptor beta 12-3
TRBV14	=	T cell receptor beta 14
TRBV23	=	T cell receptor beta 23
TRBV27	=	T cell receptor beta 27
Treg	=	Regulatory T cells
Trm	=	Resident memory T cell
T1DM	=	psoriasis and type 1 diabetes mellitus
Vα7.2	=	Variable alpha 7.2
Vβ2	=	Variable beta 2
Vβ13	=	Variable beta 13
AC-6-FP	=	Acetyl-6 formylpterin
6-FP	=	6-formylpterin
5-OE-RU	=	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	=	5-(2-oxopropylideneamino)-6-D-Ribitylaminouracil
RL-6,7-diMe	=	6,7-dimetyhyl-8-D-ribityllumazine
RL-6-Me-7-OH	=	7-hydroxcy-6-methyl-8-D- ribityllumazine

CHAPTER I

INTRODUCTION

Mucosal-associated invariant T (MAIT) cells are an innate-like T cell subset in humans. MAIT cells are unconventional T cells identified by the high expression of Vα7.2 TCR, CD161 and IL-18Rα and its restriction to the non-classical MHC class 1 related molecule or MR1**.** MAIT cells differ from conventional T cells in that MAIT cells express semi-invariant T cell receptors (TCRs), which are made up of TRAV1-2 (Vα7.2)-TRAJ33/TRAJ20/TRAJ12 paring to TCRβ chains predominantly TRBV6 family and TRBV20. MAIT cells consist of 3 subpopulations: $CD8^+$, double negative (DN) and $CD4^+$. Vitamin B metabolites are antigens of presented by MR1 to MAIT cells. MR1 ligands can be categorized into 2 groups: vitamin B2 or riboflavin and vitamin B9 or folic acid. Moreover, MAIT cells can recognize some drug and drug like molecules such as diclofenac and 5-OH-diclofenac. MAIT cell activation occurs by 2 pathways: MR1-dependent pathway by bacterial or yeast infection though MR1–ligand-TCR complex and MR1-independent pathway by viral infection and inflammatory cells through cytokine receptors. MAIT cells activation results in the production of cytokines and cytotoxic molecules including IFN-γ, TNF-α, IL-17, IL-22, perforin and granzyme B. MAIT cells are abundant in peripheral blood (1-10%) and found in gastrointestinal tract, liver, kidney, lymphoid organs, etc. MAIT cells play a role in many diseases including bacterial infection, fungal infection, viral infection and inflammatory disease.

MAIT T cell receptors (TCR) repertoire have been previously studied in peripheral blood. Several studies investigated the TRAV and TRBV gene usage including CDR3 segment. However, data on MAIT TCR repertoire in gastrointestinal tract are lacking.

Thus, we were interested to study MAIT TCR repertoire in both peripheral blood and gastrointestinal tract including duodenum, ileum and colon.

Research objective

To investigate MAIT TCR repertoire isolated from PBMCs, duodenum, ileum and colon

To compare MAIT TCR repertoire between peripheral blood and gastrointestinal tract.

Hypothesis

MAIT TCR repertoire are different in each site.

MAIT TCR repertoire in mucosal is more diverse than peripheral.

CHAPTER II

LITERATURE REVIEW

Gastrointestinal (GI) tract

The structure of the gastrointestinal (GI) tract consists of the esophagus, stomach, small intestine, large intestine and anus [1]. The average length from the mouth to anus is approximately 5 meters in humans [2] . Each structure has its own function. The esophagus is responsible for passing down food with a surface area of 0.02 m^2. The stomach has a digestive function and is permeable to macromolecules with a surface area of 0.05 m^2. The small intestine consists of three parts: (i) duodenum, (ii) jejunum and (iii) ileum and is the longest segment of the GI tract. Villi and microvilli cover the mucosal lumen of the small intestine, resulting in a very high surface area of 30 m^2. Moreover, the small intestine also has a digestive function and selectively absorbs nutrients. The large intestine (colon) absorbs remaining nutrients leftover from the small intestine with a surface area of 2 m^2 [2]. Lastly, the anus is about 4 cm long and has muscles that help passage stool out of the body (Fig. 1). Moreover, gastrointestinal tract is lined by a mucosal wall that will be explain below.

Gut mucosa

The gut mucosal wall is composed of four layers in order from the luminal to basolateral side: the mucosa, submucosa, muscle and serosa respectively (Fig. 1). The mucosal layer consists of a mucus layer, epithelial cell layer, lamina propria layer and the muscle layer, in which consist of an outer longitudinal muscle layer and an inner circular muscle layer. Contractions of the two muscle layers lead to GI wall movements during digestion and absorption (Fig. 1). In addition, the mucosal layer in the GI tract also plays a role in immune function protecting the body from microbiota and pathogens [3].

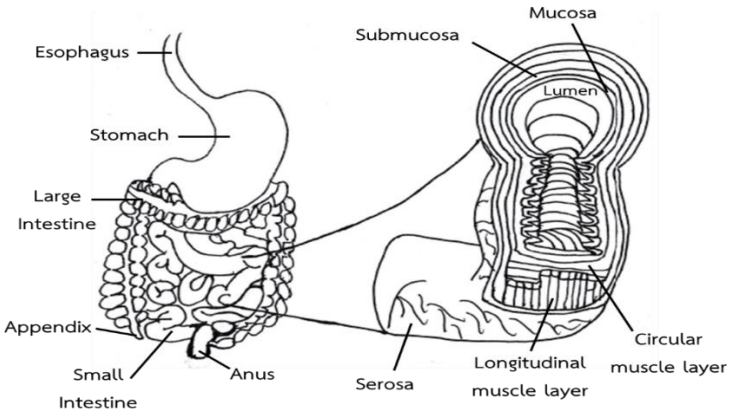


Fig. 1 Structure of the human gastrointestinal tract and the gut mucosal wall.
Left, the gastrointestinal tract composes of mouth, esophagus, stomach, small intestine, large intestine and anus. Right, cross section of the gut mucosal wall composing of four layers: mucosa, submucosa, muscle and serosa. (Adapted from https://www.britannica.com /science/brush-border)

Gut mucosal immunity

- **Innate immunity**

The innate immune system in the gastrointestinal (GI) tract has three lines of defense to infection as the followings.

1. The first line of innate immune defense is a physical barrier containing a single epithelial cell layer lining the gut lumen [4]. It functions by preventing harmful intraluminal entities including foreign antigens, microorganisms and their toxins from invading the mucosa and act as a selective filter to allow for translocation of essential dietary nutrients, electrolytes and water from the intestinal lumen into the circulation by facilitation of junctional complexes between each cell [5].

2. The second line of innate immune defense in the gut mucosa is a chemical barrier composed of molecules secreted from cells in the mucosal layer.

2.1 Mucin

Mucin is secreted from goblet cells located above the epithelial layer and plays a role in limiting epithelial cell exposure to microorganisms [6]. Its function include protection of the mucosal epithelial surface, maintenance of epithelial integrity and cell adhesion. [7]. In terms of protection, mucin plays a role in signaling, monitoring and repairing epithelial damage [8]. Mucin can also regulate microbial-mucin interactions and promote stable microbial community as a source of nutrients [9]. Many studies in mouse models show that mucus layer breakdown can lead to intestinal inflammation and disease [10-12].

2.2 Antimicrobial peptides (AMPs)

AMPs are host defense peptides that compose of cationic and amphiphilic (hydrophilic and hydrophobic) residues. AMPs are secreted from Paneth cells [13] [14]. Positively charged residues of cationic AMPs directly interact with negatively charged components of bacterial cells leading to cell depolarization, leakage of cellular contents, and cell death; whereas hydrophobic residues of hydrophobic AMPs incorporate themselves into lipid bilayers and cause membrane permeabilization and disruption, leading to cell death of bacteria [15]. Example of AMPs includine cathelicidin and defensin.

2.3 Lysozymes

Lysozymes are secreted by Paneth cells [16] and play an important role in limiting bacterial growth at mucosal surfaces and other sites. Lysozymes not only control pathogenic bacteria, but also limit overgrowth of the microbiota to prevent dysbiosis. Lysozyme hydrolysis of Peptidoglycan leads to cell wall instability and bacterial cell death. [17].

3. The third line of innate immune defense are the immune cells found in the gut mucosa.

3.1 Dendritic cells (DCs)

Dendritic cells (DCs) are bone marrow-derived antigen presenting cells. DCs can develop from both tissue resident macrophages and monocyte-derived populations. There are found in secondary lymphoid tissue, such as mesenteric lymph nodes (MLNs), Peyer's patches and lamina propria (LP). DCs can produce pro-inflammatory cytokines, Interleukin (IL)-12 and IL-23 to promote intestinal inflammation [18, 19] and cytokine type I Interferon (IFN). Moreover, DCs also promote IgA expression in the gut mucosa called "isotype switching" that occurs via both dependent and independent of T cell [20].

3.2 Macrophages

Macrophages are phagocytes differentiated from monocytes by promotion of IL-10 and TGFβ [21]. Macrophages are phenotypically either M1 being classically activated (pro-inflammatory macrophages) or M2 being alternatively activated (anti-inflammatory macrophages) [22]. Intestinal resident macrophages can be found in different layers of the gastrointestinal tract such as lamina propria, submucosa, circular muscle, longitudinal circular muscle and serosa [23]. Macrophages play an important role as professional antigen presenting cells (APCs), help maintain microbiota tolerance while still being responsive to pathogens and produce inflammatory cytokines (IL-1β, tumor-necrosis factor alpha (TNF-α), IL-6, IL-12 and IL-23) [24]. In addition, macrophages contribute to wound healing.

3.3 Innate lymphoid cells (ILCs)

Innate lymphoid cells (ILCs) are a heterogeneous immune cell population. ILCs rapidly response to pathogens infection or tissue damage through germline-encoded receptors [25]. ILCs are categorized into 3 groups; ILC1s, ILC2s and ILC3s. ILC1s produce cytokines IFN-γ, tumor-necrosis factor alpha (TNF-α) similar to Th1 [26]. ILC2s produce IL-5, IL-6, IL-9, and IL-13 and are activated upon intestinal epithelial cells (IEC)-derived IL-33, IL-25, or thymic stromal lymphopoietin (TSLP) similar to Th2 [25]. ILC3s produce IL17A and/or IL-22 similar to Th17 [27]. ILCs are important for host defense against infection, metabolic homeostasis, and tissue repair.

3.4 Polymorphonuclear cells (PMNs) are innate immune cells characterized by their polymorphic nuclei and short half-life [28]. They are recruited to the site of infection by CXC-chemokines and produce reactive oxygen species (ROS) [29] (Fig. 2). The extracellular ROS; H_2O_2 at high concentration are highly toxic to microbial defense and at lower concentrations arelong range signaling [30]. Moreover, PMNs promote inflammatory resolution and mucosal homeostasis [31].

- **Adaptive immunity**

The adaptive immunity in the GI tract is composed of cell-mediated and humoral-mediated immunity.

Cell-mediated immunity

T cells in the GI mucosa consists of intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) based on the mucosal layers [32].

1.1 Intraepithelial lymphocytes (IELs)

Intraepithelial lymphocytes (IELs) are located within the epithelial cell layer of the GI mucosa. In humans, there are about 10–20 IELs per 100 villi [33] and these cells belong to both $\alpha\beta^+$ and $\gamma\delta^+$ T cells. IELs in the mucosa can be divided into "natural" IELs and "induced" IELs. Precursors for natural IELs go through an "alternative" self-antigen based thymic maturation process [34] resulting in functional differentiation of mature CD4 and CD8$\alpha\beta$ double-negative, TCR$\gamma\delta$-expressing or TCR$\alpha\beta$ -expressing T cells that directly migrate to the intestinal epithelium. Induced IELs are predominantly shaped by non-self-antigens (foreign antigens) [35, 36]. Thus, induced IELs increase with age in response to exposure to exogenous antigens whereas natural IELs remain constant and become a minor IEL population in adult age. IELs are rapidly activated and have cytolytic and immunoregulatory effect that protect host tissues from infection, cell transformation, and uncontrolled infiltration by systemic cells such as $CD4^+$ $\alpha\beta$ T cell. Moreover, IELs contain cytoplasmic granules for cytotoxic activity, and can express IFN-γ, IL-2, IL-4, or IL-17 [27].

1.2 Lamina propria lymphocytes (LPLs)

Lamina propria lymphocytes (LPLs) are T cells located in the lamina propriae [32]. LPLs are classified into two major subsets based on T cell receptor (TCR) and coreceptor expression: the conventional T cells expressing both CD4 and CD8 $\alpha\beta$ T cells and unconventional T cells ($\alpha\beta$ T cells including natural killer T (NKT) cells and mucosal-associated invariant T (MAIT) cells [37] (Fig. 2). The role of T cells will be explained later in the thesis.

Humoral immunity

For humoral-mediated immunity in the gut mucosa, B cells and IgA play an important role [38]. B cells in the GI tract can be found in the tonsils, adenoids, Peyer's patches of the small intestine, appendix and lymphoid follicles of the large intestine and rectum [38]. In germinal centers, B cells become memory B cells or differentiate into plasmablasts, which further differentiate into plasma cells. Plasma cells can be found in the submucosa and lamina propria [38].

B cell responses in gut tissues require introduction of antigens from the gut lumen into the mucosal tissue. Oral antigens can be sampled from the lumen by microfold (M) cells that overlay Peyer's patches, by dendritic cells and macrophages, and by intestinal goblet cells and epithelial cells that express antibody receptors that can transport antigen-antibody complexes [39]. Antigens transported by M cells and dendritic cells are delivered to Peyer's patches, while antigens that are sampled by the villous epithelium by DCs and macrophages enter the lamina propria and transported to the mesenteric lymph nodes. Antibody-secreting cells (plasmablasts and plasma cells) are guided to their effector sites in the lamina propria of the GI tract, or to the blood and other tissues through expression of homing markers [40] CC-chemokine receptor 7 (CCR7), CCR9 and CCR10) [41].

Plasma cells in the lamina propria and colon predominantly express IgA [38]. IgA is the most abundant antibody which play an important role in gut mucosal immunity. Mucosal IgA is polymeric in structure, while serum IgA is monomeric [42]. Secretory IgA (sIgA) is a polymeric IgA composed of dimeric IgA, J chain, secretory component (SC) and the cleavage product of the polymeric Ig receptor (pIgR). Colonization of microbiota results in specific sIgA secretion. This contribution of sIgA in maintaining microbiota leads to a balance between appropriate neutralization and correct sensing by intestinal epithelial cells (IECs) [43] (Fig. 2).

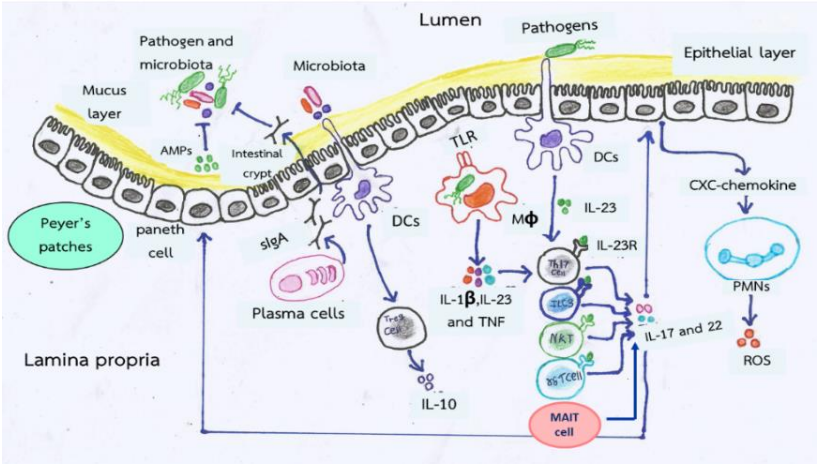


Fig. 2 General overview of the gut mucosal immunity.

Dendritic cells (DCs) sample gut microbiota and DCs induce a tolerogenic response by activating regulatory T (Treg) cells to secrete IL-10. Macrophages and DCs are activated by pathogens to secrete IL-23, which stimulate many subsets of T cells including Th17, γδ T cells Treg, NK cells NKT cells ILC3s and MAIT cells to secrete IL-17 and IL-22. These cytokines lead to CXC-chemokines secretion of intestinal epithelium that attracts neutrophils. In addition to chemokines, IL-17 and IL-22 induce the production of antimicrobial peptides (AMPs) which balance the microbial composition of the intestinal lumen. Plasma cells help control the microbiota and pathogens by secretory IgA (sIgA). (Adapted from Perez-Lopez et al., *Nature*, 2016)

Role of T cells in gut mucosal immunity

Conventional T cells in gut mucosa

T lymphocytes play a major role in the defense, memory and tolerance for tissue homeostasis [44].

1.1 CD4+ T cells

$CD4^+$ T cells produce cytokines that are critical in driving immune responses to pathogen and maintaining epithelial barrier integrity [45]. The important subsets include T helper-1 (Th1), Th2, Th17, Th22 and follicular helper T (Tfh) cells [46]. Th1 cells produce IFN-γ in response to intracellular pathogens and in viral infections

[46]. Th2 cells produce IL-4, IL-15, and IL-13 conferring protection against helminthic infection and also participate in pro-inflammatory pathways in chronic intestinal inflammatory disorders such as inflammatory bowel disease (IBD) [47]. Th17 cells can produce IL-12, IL-17, IL-22 and IL-23 in response to microbiota such as segmented filamentous bacteria (SFB) and pathogens such as *Citrobacter rodentium* [48] and promote inflammation in several autoimmune disorders [49]. Th22 cells produce IL-22 that promote epithelial cell proliferation and survival and play an important role in wound healing [50]. Follicular helper T cells (Tfh) produce IL-21 regulating B cells in germinal centers (GCs) that promote high-affinity sIgA production in response to microbiota [51].

1.2. Regulatory T cells (Treg)

Regulatory T cells (Treg) living in the intestine maintain homeostasis by balancing inflammatory effects and inducing the tolerant responses to antigens from microbiota and food [44]. This function include intestinal tolerance and Oral tolerance. The intestinal tolerance acts locally in the gut to prevent inflammatory reactions, ranging from mild allergy to anaphylaxis by suppress pro-inflammatory Th1, Th2 and Th17 immune responses [52]. In contrast, oral tolerance refers to a process which the repeated exposure to the same food antigen does not bring out an immune response and after systemic challenge with the same antigen together with a strong adjuvant [52] by suppress Th2-dependent allergic inflammation by

decreasing mast cell activation, IL-4/IL-5/IL-13 production from ILC2 and Th2 cells, and IgE production from plasma cells [53].

1.3. CD8+ T cells

Both lamina propria (LP) and epithelium have $CD8^+$ T cells. $CD8^+$ T cells are characterize into naive, effector, and memory T cells [54]. $CD8^+$ resident memory CD8 T (Trm) cells consist of conventional CD8$\alpha\beta^+$ and unconventional CD8$\alpha\alpha^+$ cells and express $CD103^+$ phenotype [55]. Because of Trm cells are not only persistent, non-circulatory cells, which provide particularly rapid and efficient protection against infections but also produce pro-inflammatory cytokines that rapidly trigger both innate and adaptive protective immune responses. Functionally, lamina propria CD8 Trm cells produce polyfunctional profile cytokine such as IFN-γ + IL-2 + TNF-α and express cytotoxic granules after stimulation such as perforin and granzyme-B, whereas epithelium CD8 Trm cells produce polyfunctional profile cytokine only IFN-γ + IL-2 [55].

Unconventional T cells in gut mucosa

2.1 $\gamma\delta$ T cells

$\gamma\delta$ T cells represent a major T cell population in epithelial surface and perform tissue homeostasis and repair. $\gamma\delta$ IELs secrete keratinocyte growth factor 1 (KGF1) which induces intestinal epithelial cell (IEC) proliferation and increases barrier

integrity and epithelial repair after tissue damage [56, 57]. γδ IELs scan the epithelium for expression of MHC I-related genes A and B (MICA and B) and MICA/B is recognized with high affinity by the natural killer (NK) cell receptor NKG2D of γδ IELs for stress-inducible triggers of γδ T cell cytotoxicity [58]. In addition, γδ IELs can produce C-type lectins regenerating islet-derived protein 3 gamma (RegIIIγ) [59] and cytotoxic granules such as granzymes A and B potential towards pathogens and infected cells [60].

2.2. Natural killer T cells (NKT cells)

Natural killer T cells (NKTs) recognize lipid antigens presented by the non-classical major histocompatibility complex (MHC) class I molecule, CD1d. Human intestinal NKT cells are mainly localized within the lamina propria [61]. iNKT cells regulate intestinal homeostasis by sensing lipids presented by $CD11c^+$ cells. iNKT cells both directly and indirectly modulate the function of other intestinal immune cells as well as the composition and stratification of intestinal bacteria [62]. CD1d-mediated crosstalk between iNKT cells and IEC modulates IL-10 secretion [63] and engagement of CD1d on group 3 ILC3 induces IL-22 production [64]. Moreover, iNKT cells can produce IFN-γ, IL-6 and IL-17 response to bacterial infection in the GI tract [65].

2.3. Mucosal-associated invariant T (MAIT) cells

Mucosal-associated invariant T cells (MAITs) recognize small metabolite molecules derived from two vitamin B sources: vitamin B2 (riboflavin) synthesized by many bacteria and yeasts, but not by mammals, which activate MAIT cells; and vitamin B9 (folic acid), which do not activate MAIT cells. MAIT cell antigen recognition is restricted to the major histocompatibility complex class 1 related molecule (MR1) [66]. In this thesis, MAIT cells will be the focus and will be discussed later.

Microbiota and mucosal immunity

After birth, the microbiota starts colonizing in the gut. The initial composition of microbiota in life is variable, but becomes relatively stable after 2 years forward into adulthood [67]. This ecosystem consists of unique features such as pH and dynamic microbe–tissue interactions for microbial biotransformation [68]. Biotransformation is the transformation of various compounds including hydrocarbons, pharmaceutical substances and metals by bacteria using bacterial enzyme [69]. The major microbiota are *Lactobacillus*, *Streptococcus* and *Enterobacteriaceae*. The highest populations of microbiota are found in the colon estimateting 10^{10}-10^{13} CFU/ml (Fig. 3). The host provides a habitat and nutrients for microbiota whilst the gut microbiota support the development of the metabolic system and the maturation of the intestinal immune system [70]. The host-microbiota interaction triggers antimicrobial responses from the epithelium including

the release of several antibacterial lectins [71, 72]. Moreover, the microbiota produce metabolites which may stimulate IgA production through class switching, leading to immune tolerance of the gut [73]. When pathogens entry to colonize at the mucosal surface, microbiota can prevent by pathogen colonization resistance process, as well as indirectly stimulation of the immune response which provides signals to stimulate the normal development of the immune system likewise the maturation of immune cells [74]. However, when balance of gut microbial communities change, it may lead to development of diseases such as inflammatory bowel diseases (IBD), systemic lupus erythematosus (SLE) and rheumatoid factor, anti-citrullinated protein antibodies (ACPAs) in rheumatoid arthritis (RA) [75, 76].

Some species of microbiota have a vitamin B synthesis abilities. For example bacteria including *Lactobacillus lactis*, *Lactobacillus reuteri*, *Lactobacillus fermentum*, *Bacteroides fragilis*, *Bifidobacterium adolescentis* and *clostridium diffic*ile have abilities to synthesize vitamin B2. *Lactobacillus pantarum*, *Bifidobacterium adolescentis*, *Bifidobacterium dentium* and *Streptococcus thermophilus* have abilities to synthesize vitamin B9 [77]. These vitamin B2 and vitamin B9 are antigens for MAIT cells activation.

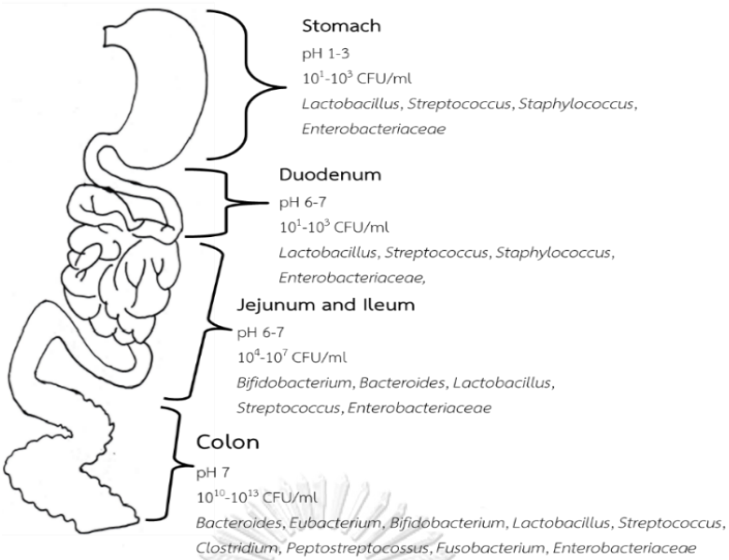


Fig. 3 Localization and abundance of microbiota along the human gastrointestinal tract (adapted from Clarke et al., *Pharmacol* Rev. 2019)

Mucosal-associated invariant T (MAIT) cells

MAIT cell phenotype

MAIT cells are an innate-like T cell subset that is distributed in the blood and mucosal sites. They are abundant in peripheral blood (1-10%) and found in liver, kidney, lymphoid organs (tonsils and lymph nodes), ovaries, prostate, adipose tissue and the skin [78]. MAIT cells have 3 subpopulations: $CD8^+$, $CD4^-CD8^-$ double negative (DN) and $CD4^+$ MAIT cells [79].

MAIT cells are identified by their high expression of Vα7.2 TCR, CD161 and IL-18Rα and their restriction to the non-classical MHC class 1 related molecule or MR1 [80]. Moreover, MAIT cells also express chemokine receptors CCR2, CCR6, CXCR6 and CCR5 as their tissue homing marker, cytokine receptors IL-2Rβ, IL-7Rα, IL-12R, IL-18Rα, IL-23R, IFN-αR and transcription factors C/EBPδ, T-bet, EOMES, Blimp-1,

RORγt PLZF and STAT3 and the C-type lectin-like protein NKG2D [78, 81] (Fig.4) However, a study has found that some MAIT cells were Vα7.2 negative population [82]. This Vα7.2 negative population called "non-classical MAIT cells" exhibit antigen restriction and phenotypic features similar to Vα7.2 positive MAIT cells and express other TRAV and TRBV [83].

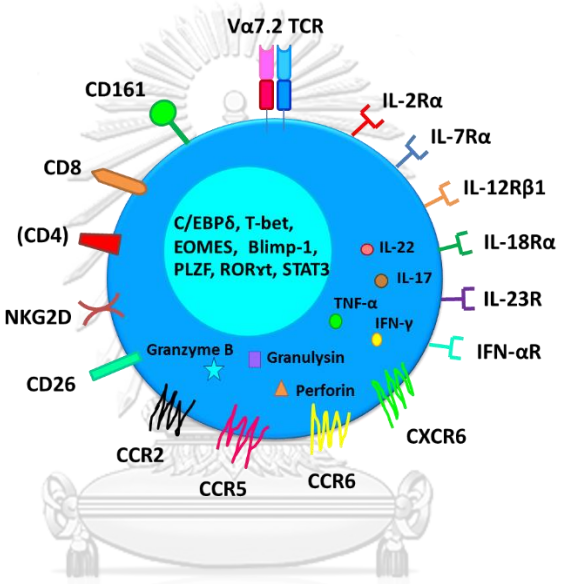


Fig. 4 Human MAIT cells phenotype.

Human MAIT cells are characterized by expression of Vα7.2 and CD161, transcription factors C/EBPδ, T-bet, EOMES, Blimp-1, RORγt PLZF and STAT3, co-receptor CD8 or CD4, cytokine receptor IL-2Rβ, IL-7Rα, IL-12R, IL-18Rα, IL-23R and IFN-αR, chemokine receptor CCR2, CCR6, CXCR6 and CCR5, and the C-type lectin-like protein, NKG2D. (Adapted from Reantragoon et al., *Asian Pac J Allergy Immunol*, 2016 and Provine et al., *Annu Rev Immunol* ,2019)

MAIT cell antigen recognition

MAIT cells recognize vitamin B metabolites that is presented by the non-classical MHC class I-related (MR1) molecule [84]. MR1 ligands are categorized into 2 groups. The first group is vitamin B2 or riboflavin, which bind to MR1 as agonists, such as monocyclic pyrimidine derivatives (eg. 5-OP-RU and 5-OE-RU) and bicyclic lumazines (eg. RL-6,7-DiMe and RL-6-Me-7-OH). The second group is vitamin B9 or

folic acid, which bind to MR1 as antagonists, such as bicyclic pterins (eg. 6-FP and Ac-6-FP) [85, 86]**.** Moreover, MAIT cells can recognize some drug and drug like molecules such as diclofenac, 5-OH-diclofenac and 4′-OH-diclofenac [87] (Fig. 5).

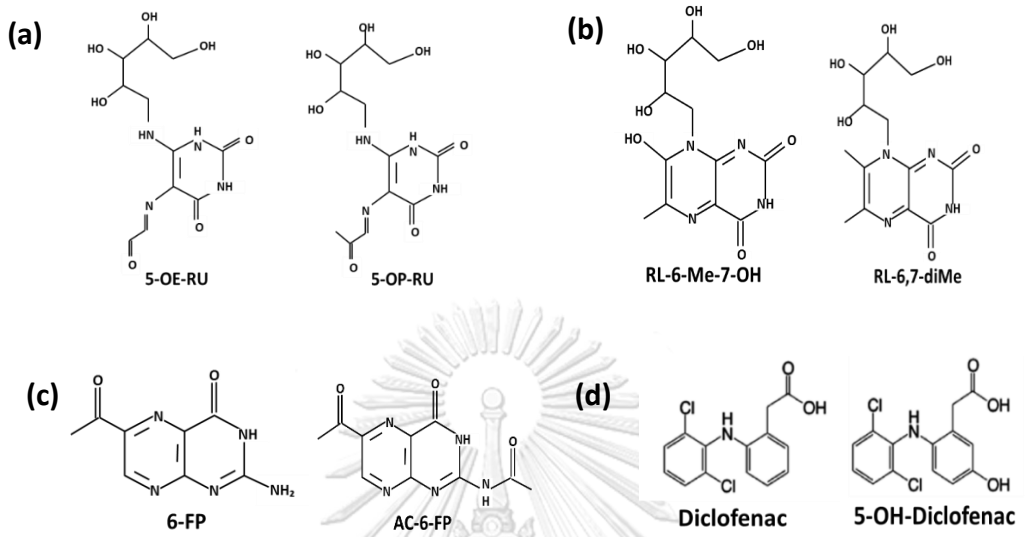


Fig. 5 Structure of MR1 ligands.
(a) monocyclic pyrimidine derivatives (b) bicyclic lumazines (c) bicyclic pterins. (d) Drug and drug like molecule (MR1 ligand adapted from McWilliam et al. *Current Opinion in Immunology*, 2015)

MAIT cell activation

MAIT cell activation occurs by 2 pathways. The first pathway is MR1-dependent activation by bacteria that produce vitamin B metabolites loaded onto MR1 and presented on the surface of infected cells. The semi-invariant MAIT T cell receptor binds to the MR1–ligand complex resulting in MAIT cell activation.

The second pathway is MR1-independent activation by cytokines produced by infected cells. These cytokines include interleukin-12 (IL-12), IL-15, IL-18 and IL-7 and activate MAIT cells through stimulation of cytokine receptors [88]. MAIT cells can be activated by IL-12 and IL-18 which are produced by inflammatory cells in non-

infectious diseases or infected cells in viral diseases. In addition, MAIT cells also respond to some bacterial infections such as *Mycobacterium tuberculosis*, *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), and *Enterococcus faecalis*, in an MR1-independent manner [89]. BCG-induced activation of MAIT cells in tuberculosis (TB) patient was mostly mediated by IL-12 and IL-18 compared to IL-2, IFN-α, suggesting that activation of MAIT cells in an MR1-independent manner is a major mechanism for IFN-γ production response in *Mycobacteria* infection [90].

The activation of MAIT cells results in the production of Th1 and Th17 cytokines; and release of perforin and granzyme B to directly kill infected cells [91] (Fig. 6)

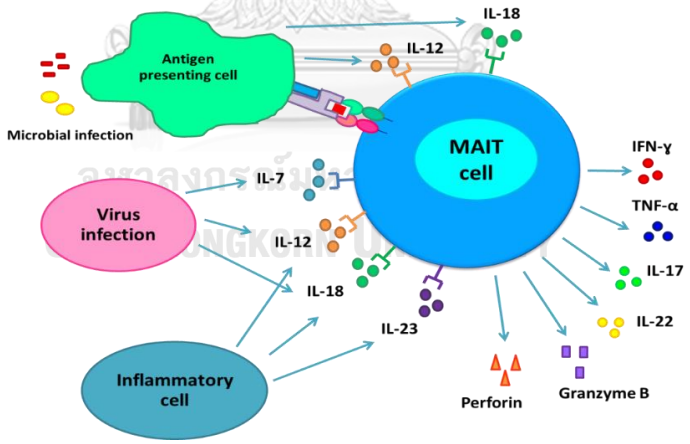


Fig. 6 MAIT cell activation.
MR1-dependent pathway by bacterial or yeast infection though MR1–ligand complex and MR1-independent activation by viral infection and inflammatory cells via cytokine receptor

MAIT cells and disease

MAIT cells play a role in infectious disease such as

1. Bacterial infections such as *Helicobacter pylori [92] E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Lactobacillus acidophilus, Staphylococcus aureus, Staphylococcus epidermidis, Mycobacterium tuberculosis* and *Salmonella enterica* serovar Typhimurium [93],

2. Fungal infections such as *Candida albicans, Candida glabrata,* and *Sacchromyces cerevisiae* [93],

3. Viral infections such as human immunodeficiency virus (HIV), influenza virus, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections) [94],

4. Inflammatory diseases such as autoimmune diseases (multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis and type 1 diabetes mellitus (T1DM); malignant diseases and inflammatory bowel disease (IBD) [95].

MAIT T cell receptor (TCR)

MAIT cells are $\alpha\beta$ T cells that possess a T cell receptor (TCR) with limited diversity characterized by an invariant α chain, Vα 7.2; paired to Jα33, Jα12 or Jα20. The α chain pairs with the β chain, predominantly Vβ13 and Vβ2. The diversity of the MAIT TCR derives from DNA rearrangement as discussed below.

TCR rearrangement

The TCR consists of a variable (V), diversity (D), joining (J) and constant (C) region in each chain that are derived from DNA rearrangements of V-D-J gene segments. Different combinations of V-D-J gene segments produce TCRs with different antigen specificities leading to diversity of the TCR repertoire. The TCRα chain compost of V, J and C segments, whereas the TCRβ chain is composed of an additional D segment apart from to V, J and C segments [96]. The process occurs by D-to-J joining before a V segment is joined to the rearranged D-J segments. Lastly, C joins to V-D-J segment and V-D-J-C segment were transcription and translation [97]. After that TCRα chain combines with the TCRβ chain resulting in formation of the $\alpha\beta$ TCR that is expressed on T cell [96].

Complementarity-determining region (CDRs)

The complementarity-determining region (CDR) is a junctional region between V, D, and J gene segments and is composed of three CDRs; CDR1, CDR2 and CDR3 (Fig 7). CDR1 and CDR2 are found in the V region and CDR3 includes a partial segment of V region, all of J region and a partial segment of D region [98]. The CDR1 and CDR2 regions on the variable region interact with MHC molecules whilst the CDR3 region interacts with the MHC-bound antigen [99]. The CDR3 is the region most affected by recombination and most variable region within a TCR in both the α and β chain. The

specificity and diversity of the TCR α- and β-chains are generated by these variable regions, especially by the CDR3 [100] (Fig. 7).

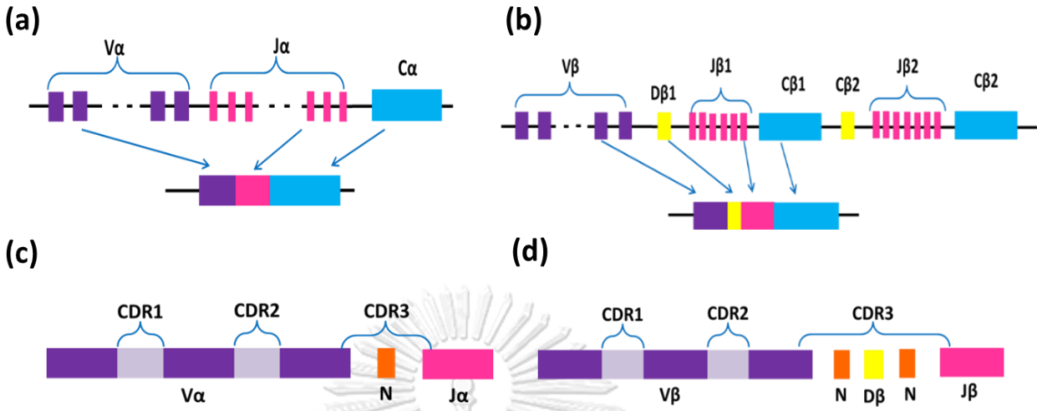


Fig. 7 T-cell receptor gene rearrangements.
(a) TCR α chain. (b) TCR β chain, with an additional diversity (D) region. (c) CDR α chain. (d) CDR β chain.

MAIT cells thymic selection

MAIT cells develop within the thymus. The positive selection of MAIT cells were occurred by MR1-expressing DP cortical thymocytes [101]. Recent studies suggest that MAIT cells may be selected by some of endogenous antigens expressed within the thymus, or by ligands from microbiota [102]. Base on the evidence of endogenous antigen presentation to MAIT cells [103], it is possible that MAIT cell selection are associate with an endogenous ligands. However, these endogenous ligands have not yet been identified [104]. In contrast, there is studies show that rapidly expansion of thymic MAIT cells population when germ-free mice were colonized with *Escherichia coli* strains, suggesting that that microbiota could be

important for MAIT cells development [105]. Thus, theses selection lead to semi-invariant of MAIT cells.

MAIT cells development

MAIT cell development occurs in the thymus [104]. Double positive MAIT cells ($CD4^+CD8^+$ MAIT cells) are positively selected by ligands presented by MR1-expressing thymocytes [106]. However, negative selection of MAIT cells has not been studied yet. Differentiation of thymic MAIT cells was investigated by Koay et al., 2018, showing that there are three-stage in MAIT cell development; (Stage 1 expressing $PLZF^-$, $CD27^-CD161^-$ and $CD218^-$; Stage 2 expressing $PLZF^{-/lo}$, $CD27^+CD161^-$ and $CD218^-$; Stage 3 expressing $PLZF^+$, $ROR\gamma t^+$, $T\text{-}bet^+$, $CD27^{+/lo}$, $CD161^+$ and $CD218^+$). Moreover, they found that co-receptor expression of MAIT cells at stage 1 and 2 were $CD4^+$, $CD8^+$ or $CD4^+CD8^+$ and stage 3 was $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ [107].

MAIT TCR receptor and MRI ligand

TCRs recognize vitamin B metabolite that are present on MR1 of antigen presenting cells [86] and these MR1-ligand complexes are bound to the TCRs by the CDR regions [98]. Thus, characterization of MAIT TCRs and CDR3 provides the basis of understanding of MAIT cells and MR1 ligand.

Rationale

MAIT cells can rapidly response to infection and predominant in mucosal gastrointestinal tract [108] Moreover, MAIT cells play important role in mucosal immunity [109]. We hypothesized that antigen are involved in clonal selection of MAIT cell. In contrast, MAIT TCR repertoire may reflect the specificity of TCR to antigen. Thus, we determine sequence of TCRs for understand both MAIT TCR and antigen. Investigation of MAIT TCRs will give an insight on understanding the nature of MAIT cells and the different MR1 ligands for use as cell therapeutic in the future. So, in our study, we aim to investigate MAIT TCR isolated from duodenum, ileum, colon and PBMC.

Research question

What is the MAIT TCR repertoire in the gastrointestinal tract site (duodenum, ileum and colon) and peripheral blood?

Hypothesis

MAIT TCR repertoire are different in each site.

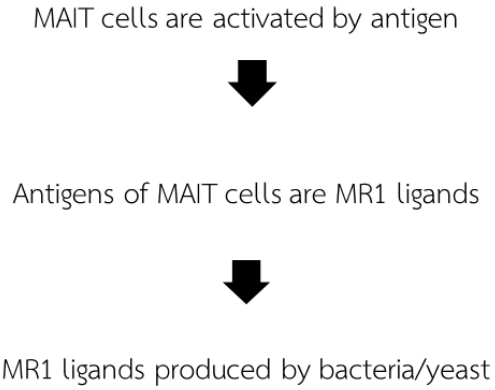
MAIT TCR repertoire in mucosal is more diverse than peripheral.

Objective

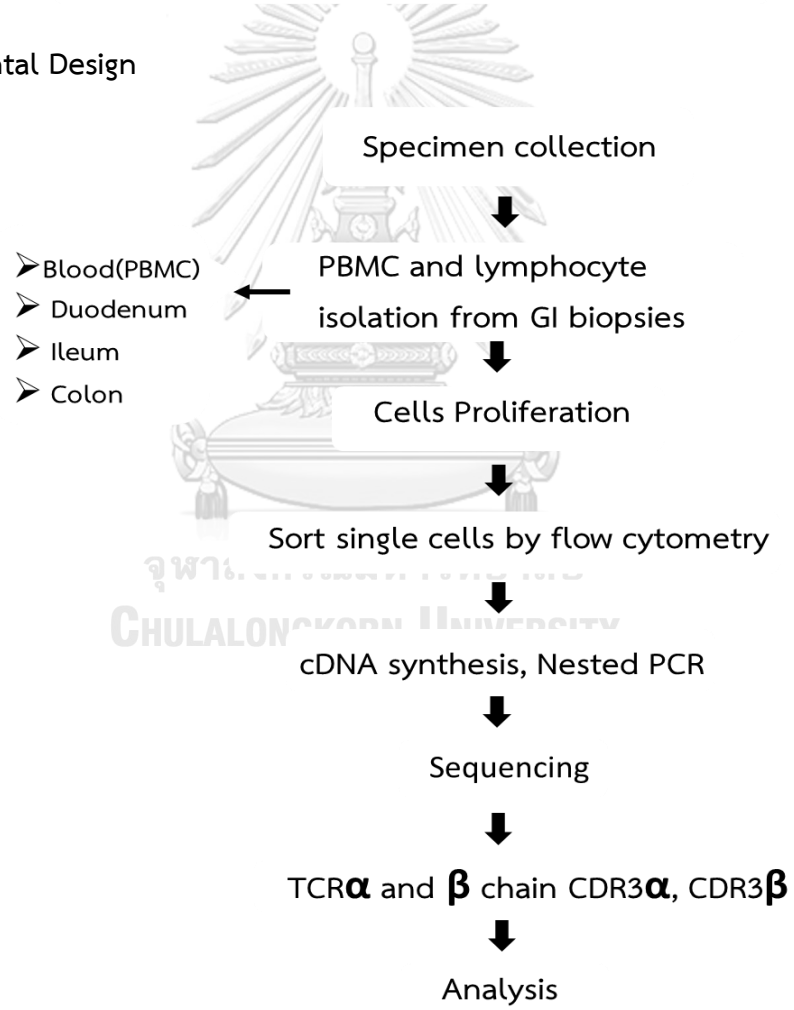
To investigate MAIT TCR isolate from PBMC, duodenum, ileum and colon.

To compare MAIT TCR repertoire between peripheral blood and gastrointestinal tract.

Conceptual framework



Experimental Design



CHAPTER III

MATERIALS AND METHODS

Blood and tissue sample collection

After obtaining informed consent (IRB number 400/60) for patients undergoing esophagogastroduodenoscopy (EGD) and colonoscopy; peripheral blood, biopsies of stomach, duodenum, terminal ileum and colon were collected from 9 individuals at (20-60 years old).

Specimen from 9 individuals were used as the followings.

Patient	Results/problems
001	Fail to proliferate.
002	**Optimized and successful proliferation and used to study TCR repertoire in this study.**
003	Cells are very low or died.
004	Cannot collect duodenum and ileum biopsies.
005	Cannot collect duodenum and ileum biopsies.
006	Cannot collect ileum biopsies.
007	Cells are very low or died.
008	Cells are very low or died.
009	**Successful proliferation and used to study TCR repertoire in this study.**

PBMC Isolation from peripheral blood

10 ml of peripheral blood from recruited patients who underwent EGD and colonoscopy were collected and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare). Blood was layered on Ficoll-Paque using a serological pipette and centrifuged at 1,500 rpm, 25°C, maximum acceleration, no deceleration for 20 minutes. PBMCs were aspirated and transfered to a new 50 ml conical tube with 10 ml of RPMI 1640 (Gibco). Cells were washed 3 times to remove Ficoll-Paque and resuspended in freezing media (90% fetal bovine serum + 10% DMSO) and preserved in liquid nitrogen until experiments were performed.

Mononuclear cell isolation from gastric and intestinal biopsies

Biopsies from the stomach, duodenum, ileum and colon were collected in 5% FBS in cold PBS and transferred to a new tube and washed with 5% FBS in cold PBS for 20 minutes to remove blood and debris. Biopsies were then incubated and gentle stirred in 5% FBS in cold PBS for one hour at 37°C to release intraepithelial lymphocytes (IELs). Cells were allowed to settle and filtered using a 70 μM strainer. The lamina propria lymphocytes (LPLs) cell fraction was isolated by further digestion with collagenase IV for 2 hours at 37°C with gentle stirring. Cells were then filtered using a 70 μM strainer and centrifuged. Pellets were resuspended with freezing media and preserved in liquid nitrogen until experiments were performed.

Cell proliferation

10^5 cells/well was plated and stimulated with anti-CD3/anti-CD28 beads. On days 3 and 5, IL-2 was added at a final concentration of 40 ng/ml. On day 7, cells were counted and surface-labeled with anti-human CD3-PE antibody (Biolegend), anti-human CD161-APC antibody (Biolegend) and anti-human Vα7.2FITC antibody (Biolegend). A 2x antibody cocktail was prepared as detailed below to label cells.

	Volume(µl) 1x	Volume(µl) 2x
anti-human CD3-PE antibody	0.5	1
anti-human CD-161-APC antibody	2	4
anti-human Vα7.2-FITC antibody	1	2
FACs buffer	46.5	93
Total Volume	50	100

Single color control was prepared as detailed below.

	Antibody (µl)	FACs buffer (µl)
anti-human CD3-PE antibody	1	50
anti-human CD8-APC antibody	1	50
anti-human CD3-FITC antibody	1	50
Unstained	0	50

Cells were transferred into a V-bottom plate and centrifuged at 1,500 rpm at 4 ºC for 5 minutes. 50 µl of surface staining antibody cocktail was added to each sample and incubated at 4ºC (on ice) for 20 minutes in the dark. Cells were washed twice using 150 µl of FACS wash buffer and centrifuged at 1,500 rpm at 4 ºC for 5

minutes and resuspended in FACS fix and acquired on a BD LSR II flow cytometer (BD Biosciences). Data was analyzed using FlowJo software v. 10 (FLOWJO, LLC).

Cell sorting

Cells were transferred into RPMI 1640 (with supplement) and centrifuged at 1500 rpm, 4°C for 5 minutes. Cells were surface-labeled with anti-human CD3-PE/Cy7 antibody (Biolegend), anti-human CD161-APC antibody (Biolegend) and anti- human Vα7.2-FITC antibody (Biolegend). A 2x antibody cocktail was prepared for total cells as detailed below.

	Volume(µl) 1x	Volume(µl) 2x
anti-human CD3-PE/Cy7 antibody	0.5	1
anti-human CD161-APC antibody	2	4
anti-human Vα7.2-FITC antibody	1	2
FACs buffer	46.5	93
Total Volume	50	100

Single color control was prepared as detail below.

	Antibody (µl)	FACs buffer (µl)
anti-human CD3-PE/Cy7 antibody	1	50
anti-human CD8-APC antibody	1	50
anti-human CD3-FITC antibody	1	50
unstained	0	50

Cells were transferred into a 96 V-bottom plate and centrifuged at 1,500 rpm at 4 ºC for 5 minutes. 50 µl of surface staining antibody cocktail was added into each sample and incubated at 4ºC (on ice) for 20 minutes in the dark. Cells were washed

twice by adding 150 µl of FACS wash buffer and centrifuged at 1,500 rpm at 4 ºC for 5 minutes and supernatant discarded. Cells were sorted using a BD FACSAria II flow cytometer (BD Biosciences) into a 96-well semi-skirt PCR plate. PCR plate were stored on dry ice during sorting and frozen at -80 ˚C until experiments were performed.

cDNA synthesis

PCR plates containing sorted single cells were thawed at 4ºC, centriguged at 1500 rpm, 4ºC and kept on ice. Reverse transcription was performed using Superscript VILO cDNA Synthesis Kit (Invitrogen). A 50x reaction mix was prepared for 40 samples as detailed below.

	Volume(µl) 1x	Volume(µl) 50x
5x VILO (Invitrogen)	0.5	25
10x Superscript (Invitrogen)	0.25	12.5
1% triton X-100	0.275	13.75
DNase RNase free water (Invitrogen)	1.5	75
Total Volume	2.5	125

2.5 µl of VILO reaction mix was added to each well. Plates were capped and pulsed with a 1500 rpm spin before incubating in a PCR thermal cycler at 25˚C for 5 min, 42 ˚C for 30 min, 80˚C for 5 min followed by plates being held at 4 ˚C.

Nested PCR

cDNA products were subjected to two rounds of nested PCR. The first round was external amplification to obtain both α and β chain using external sense Vα and Vβ an antisense Cα and Cβ segment. A 50x reaction mix was prepared for 40 samples as detailed below.

	Volume(µl) 1x	Volume(µl) 50x
10x PCR buffer (Invitrogen)	2.5	125
50 mM $MgCl_2$ (Invitrogen)	0.75	37.5
dNTP (Invitrogen)	0.5	25
TRAV external (IDT)	1	50
TRAC external (IDT)	1	50
TRBV external (IDT)	1	50
TRBC external (IDT)	1	50
Taq DNA (Invitrogen)	0.15	50
DNase RNase free water (Invitrogen)	14.6	730
Total Volume	22.5	1,125
Including template cDNA 2.5 µl = 25 µl /well		

22.5 µl of reaction mix was added to cDNA 96 well semi-skirt PCR plate (NEST) and incubated in a PCR thermal cycler (SimpliAmp Thermal Cycler, Thermo Fisher) at 95°C for 2 min, followed by 35 cycles of 95°C for 20 sec, 52°C for 20 sec and 72°C for 45 sec, and the last 1 cycle of 72°C for 7 min and held at 4°C. Plate was pulsed at 1500 rpm before and after amplification. 1-5 µl of external PCR product was amplified for TRBV usage in a new 96-well semi-skirt PCR plate (NEST). A 50x reaction mix was prepared for 40 samples as detailed below.

	Volume(µl) 1x	Volume(µl) 50x
10x PCR buffer (Invitrogen)	2.5	125
50 mM $MgCl_2$ (Invitrogen)	0.75	37.5
dNTP (Invitrogen)	0.5	25
TRBV internal (IDT)	1	50
TRBC internal (IDT)	1	50
Taq DNA (Invitrogen)	0.15	7.5
DNase RNase free water (Invitrogen)	18.1-14.1	905-705
Template (1^{st} round PCR product)	1-5	(add individual 1 µl in each well)
Total Volume	25	1,250

20-24 µl of reaction mix and 1-5 µl of template was added into each well. Plate was incubated in a PCR thermal cycler as previously described. PCR products was confirmed via gel electrophoresis.

Gel electrophoresis

2% agarose (Seva) gel was prepared with TAE buffer, SYBR Safe (Invitrogen) was mixed in agarose gel at a concentration of 1 µl per 10 ml of agarose gel solution. Samples were loaded on to the gel dye and electricity at 100 volts was applied for 30 minutes. DNA products were visualized under UV light. Both successfully amplified α and β chain were selected for sequencing. Unincorporated primers and dNTPs were removed using illustra ExoProstar 1-Step (GE Healthcare) and samples were subject to sequencing with MACROGEN, Korea.

Sequence Analysis

Data was analyzed on http://www.imgt.org IMGT/V-QUEST was selected to analyze. Species Homo sapiens (human) and receptor type (TRA/TRB) were selected and sequence were compared to data base.

Motif graph creation

The amino acid sequence was created on http://www.cbs.dtu.dk/biotools/Seq2Logo. The length of amino acid sequences was counted before and asterisks were added equaling to the longest sequence as example below.

CASSRDGREWYF*******

CASSPGDRESTQYF*****

CASSQDWSEAYEQYF****

CAWSVWQGDLSDTQYF***

CASSRPDRDRVNYQPQHF*

CATSDFWVTDPTSTDTQYF

Shannon was selected for logo type. Other setting was selected as follow: Clustering method was none, Specify threshold for clustering (Hobohm1) was 0, Weight on prior was 0, Select information content units was Bits and Output Formats was PDF.

CHAPTER IV

Results

MAIT cells can proliferate in vitro

Due to the low number of MAIT cells in each gastrointestinal tract site and being insufficient for experiments, we proliferated MAIT cells to increase MAIT cells population. We stimulated PBMCs with anti-CD3/anti-CD28 beads in the presence of IL-2 at a final concentration of 40 ng/ml at day 1, 3, 5 and 7. We evaluated the increase in cell population via flow cytometer. The results show that the number of total PBMCs and MAIT cells stimulated with anti-CD3/anti-CD28 beads and IL-2 increased from day 0 by about 9 fold, suggesting that MAIT cells can proliferate *in vitro* (Figure 8B) The population of MAIT cells in condition when stimulated at day 0, unstimulated at day 7 and stimulated at day 7 have similar levels of percentage of MAIT cells, suggesting that MAIT cells increase proportionally with other lymphocytes. Moreover, when we focused on MAIT cell subpopulation there were more CD8 MAIT cells than CD4 and $CD4^{-}CD8^{-}$ (double negative) MAIT cells both before and after proliferation, suggesting that, after proliferation MAIT cell subpopulation remained similar to the proportion prior to proliferation (Fig 8A). So, after we optimize MAIT cells proliferation in PBMC, Thus, we used these condition to proliferate lymphocytes isolated from gastrointestinal tissue biopsies to expand the number of MAIT cells.

We used this condition to proliferate mucosal MAIT cells. All lymphocytes isolated from biopsies of duodenum, ileum and colon proliferated and is demonstrated with flow cytometry after proliferation. Our results show that the percentage of MAIT cells in the duodenum, ileum and colon were 5.94, 5.32 and 5.04, respectively. Moreover, when we focused on the MAIT cell subpopulation, there were more $CD8^{+}$ MAIT cells than $CD4^{-}CD8^{-}$ (double negative) and $CD4^{+}$ MAIT

cells at every site. Our experiments was performed based on the fact that the proliferation was polyclonal and that T cell proliferation occurred homogenously.

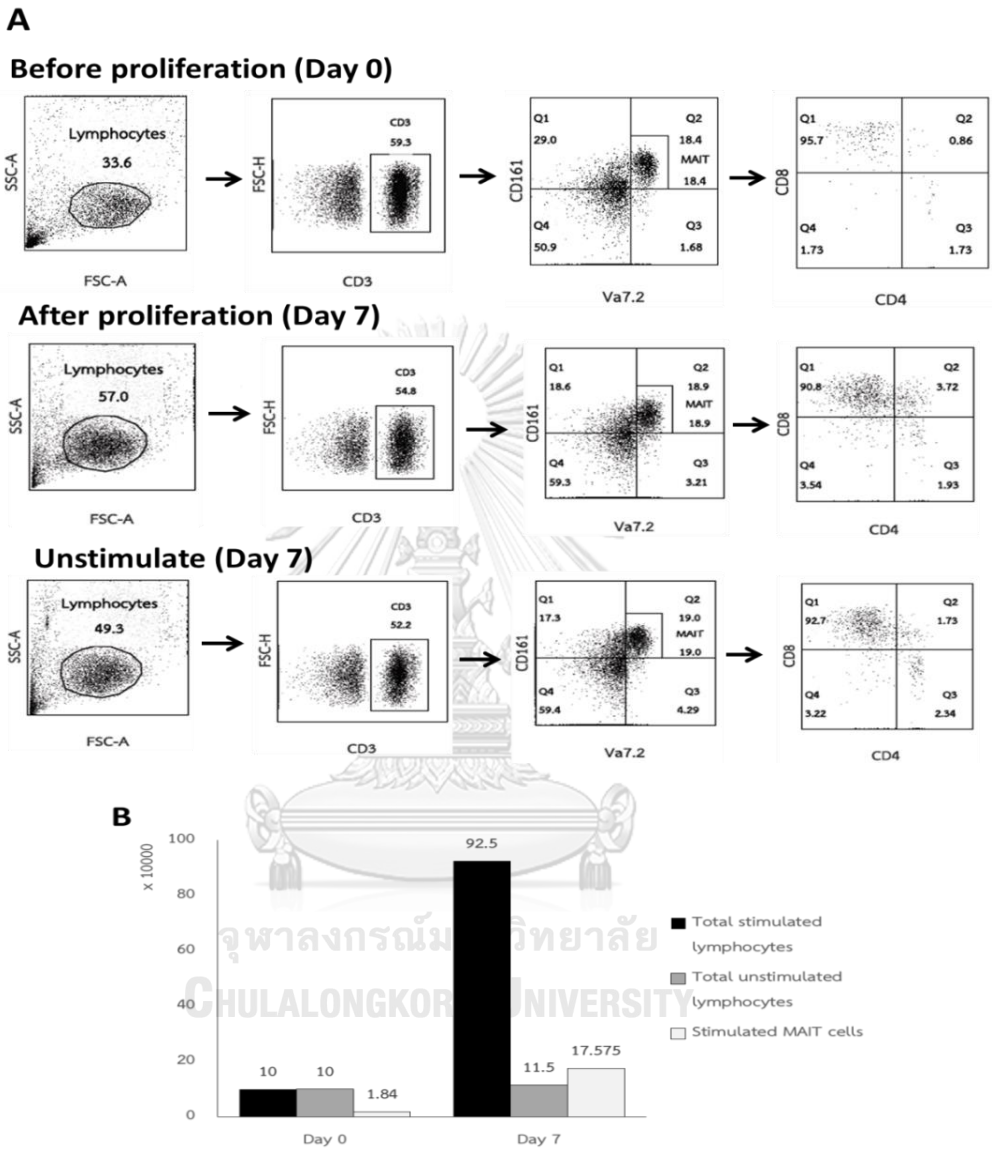


Fig. 8 Peripheral blood MAIT cell proliferation.

(A) Flow cytometric plots gating for identification of MAIT cells. Population of MAIT cells of before proliferation, after proliferation and day 7 of unstimulated were gated from $CD3^+$ T cell. Vα7.2 TCR on the X-axis and CD161 on the Y-axis were gated for MAIT cell population. Upper panel is before proliferation, middle panel is and after proliferation and lower panel is day 7 of unstimulated. (B) Bar graph comparing the number of total cells and MAIT cells prior proliferation (day 0) and after proliferation (day 7) and number of MAIT cells calculate from $\frac{\%\ \text{MAIT cell}}{100} \times$Total cells. The sample uses in this study are PBMCs.

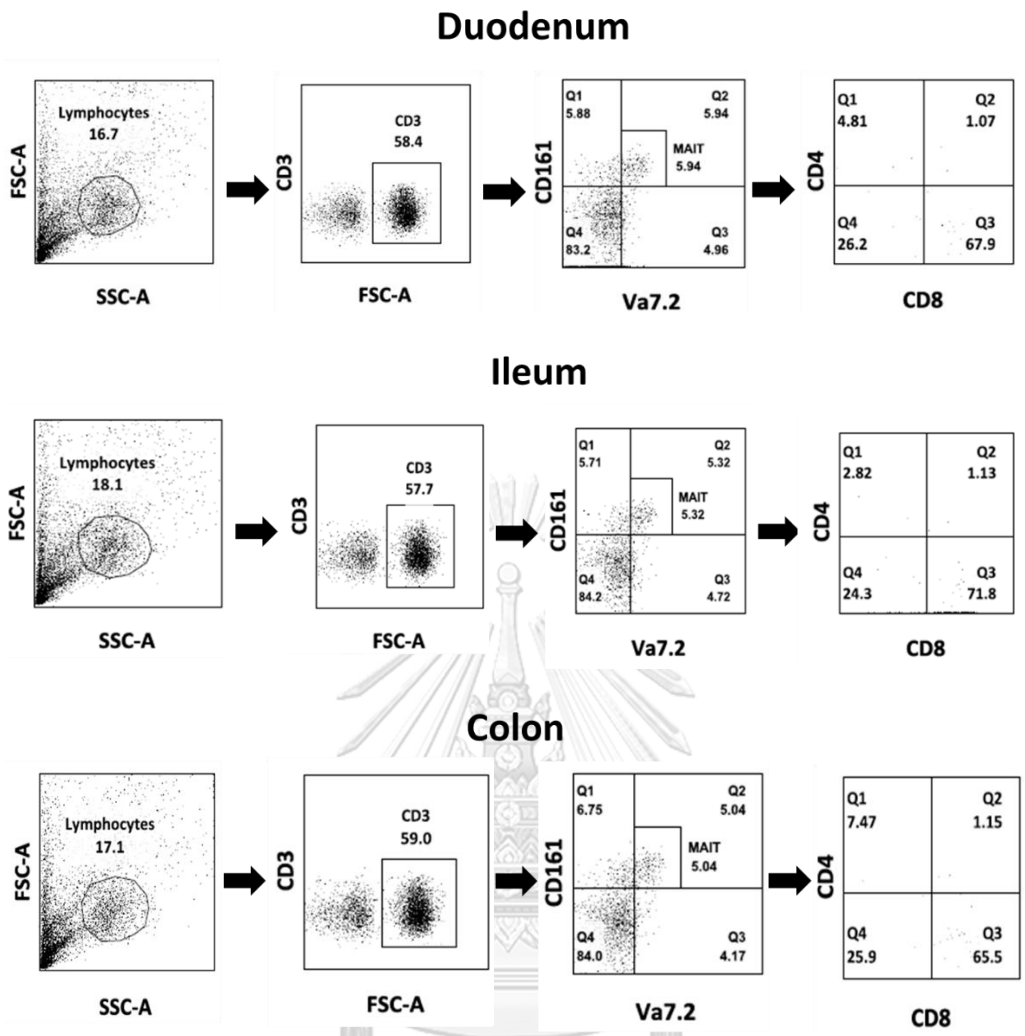


Fig. 9 Mucosal MAIT cells proliferation.

Flow cytometric plots gating for identification of MAIT cells in the duodenum, ileum and colon. Population of MAIT cells after proliferation were gated from $CD3^+$ T cell. Vα7.2 TCR on the X-axis and CD161 on the Y-axis were gated for MAIT cell population.

TCR repertoire analysis of MAIT cells from peripheral blood and gastrointestinal tract

In order to investigate the TCR repertoire of MAIT cells in peripheral blood and gastrointestinal tract, isolated PBMCs and proliferated gastrointestinal lymphocytes were single-cell sorted via flow cytometer. Next, MAIT TCRs were amplified by multiplex PCR.

An example of successful amplification of TCRα and TCRβ chain product is shown in Figure 10. This figure shows the α-chain and β-chain of MAIT TCR.

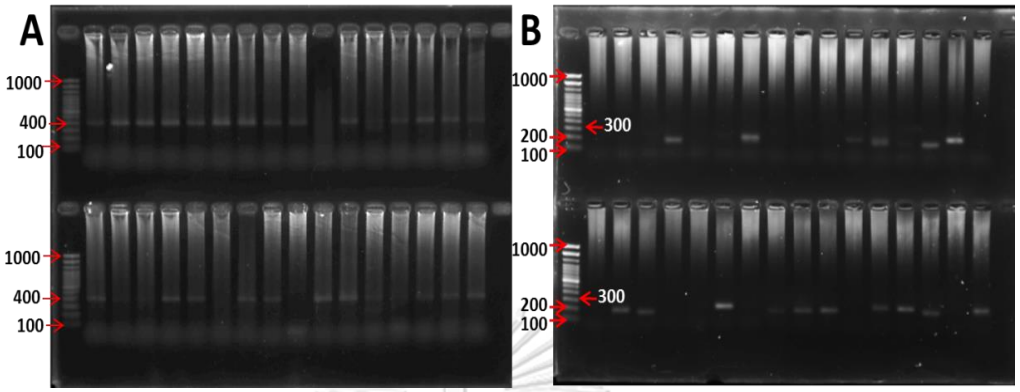


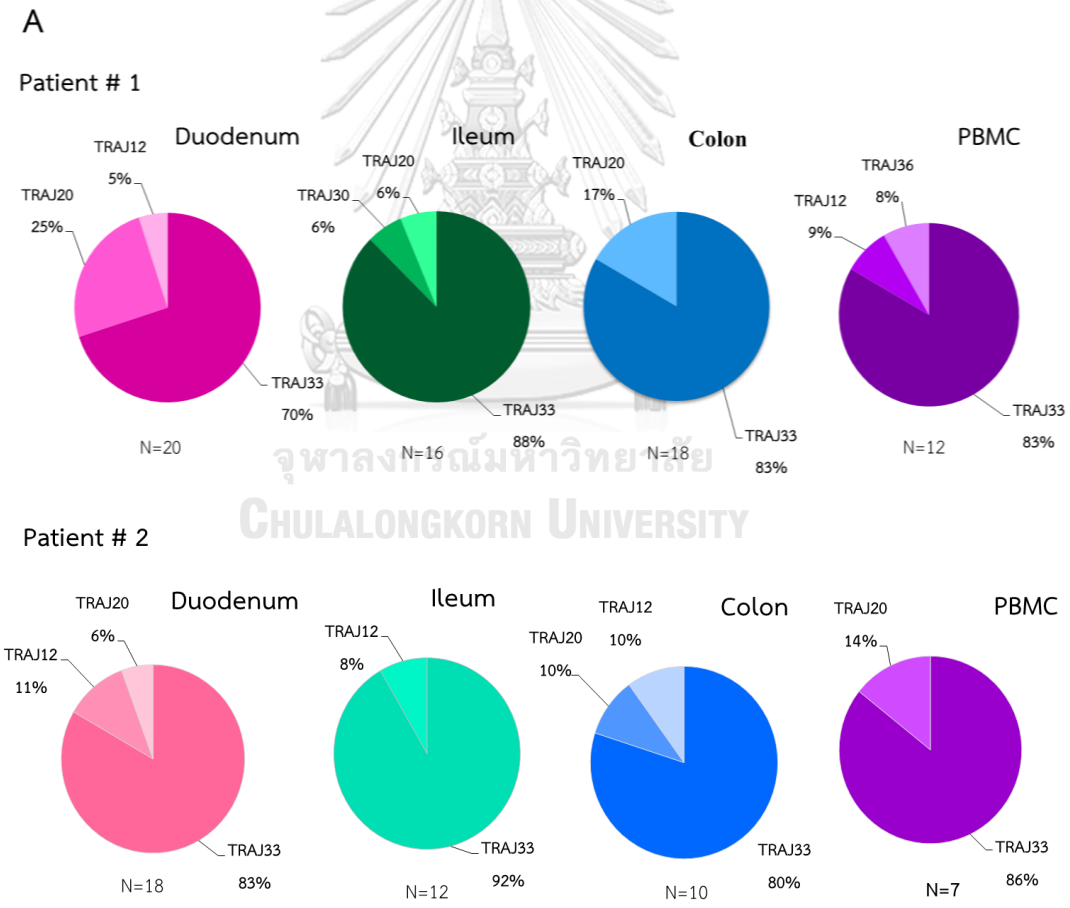
Fig. 10 Gel electrophoresis demonstrating PCR product.
from (A) Agarose gel visualization of α-chain (400 bp). (B) Agarose gel visualization of β-chain (150-300 bp).

Next, we investigated the TCR repertoire usage of MAIT cells of 2 patients. Our results show that MAIT TCRs in both patients commonly used TRAJ33 rearrangement with TRAV1-2 followed by TRAJ20 and TRAJ12, respectively. However, it was found that TRAJ30 and TRAJ36 in patient # 1 (Figure 11A). TRBV gene usage MAIT cells in both patients, most TRBV gene usage in duodenum, ileum, colon and PBMC were TRBV6-4, TRBV6-1 and TRBV20-1. (Figure 11B).

Our result show that the TRBV gene usage in duodenum, ileum, colon and PBMC of 2 patient were oligoclonal that have a small number of predominant TRBV chain. There were 10-17 TRBV chains of TRBV in each site, in which 3-4 chains were predominant. In patient # 1 we found TRBV6-1, TRBV6-4 and TRBV20-1 predominant, while patient # 2 we found TRBV4-2, TRBV6-4, TRBV20-1 and TRBV30 predominant. Moreover, there are both overlapping and non-overlapping TRBV gene usage between patient # 1 and patient # 2. Overlapping TRBV usage includes TRBV2, TRBV3-1, TRBV6-4, TRBV19 TRBV20-1 and TRBV28. Non-overlapping TRBV usages include: patient # 1 using TRBV5-8, TRBV6-6, TRBV7-2, TRBV7-8, TRBV7-9, TRBV10-1

and TRBV12-3, whereas patient # 2 using TRBV3-2, TRBV11-1, TRBV14, TRBV23, TRBV27 and TRBV30 (Fig. 11).

We compered the TRAV and TRBV gene usage of mucosal MAIT cells and peripheral MAIT cell. Our results show that TRAV1-2 of mucosal MAIT cells was pairing with TRAJ33, TRAJ20, TRAJ12 and TRAJ30, whereas TRAV1-2 of peripheral blood was pairing with TRAJ33 and TRAJ36. In addition, we found 10-17 TRBV chain of mucosal MAIT cells, whereas, 10-12 chain of peripheral MAIT cell. Moreover, the ileum in both patients was the most diverse. Thus, our results suggest that mucosal MAIT cells was more diverse than peripheral MAIT cell (Fig. 12).



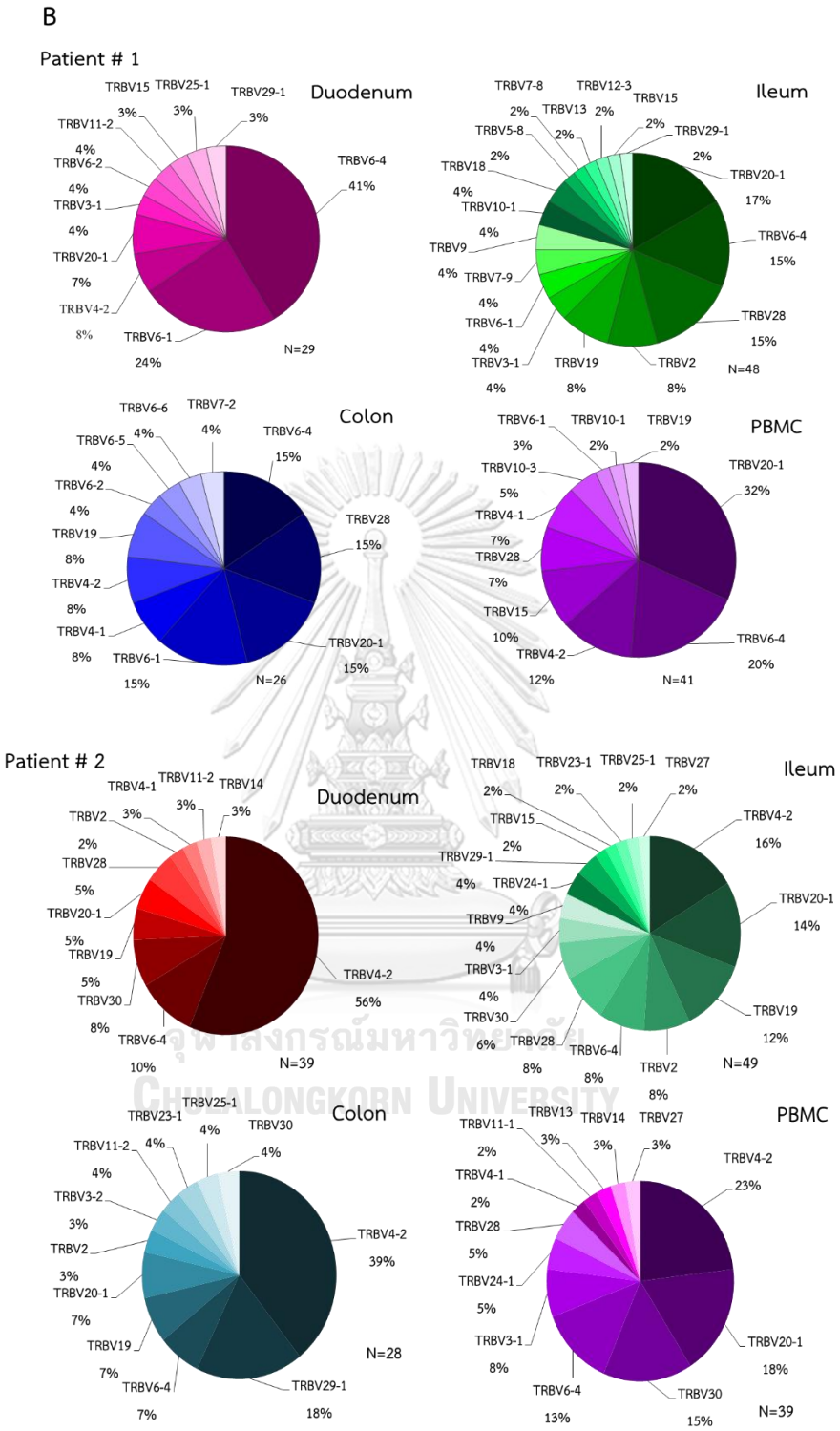


Fig. 11 TCR repertoire usage of MAIT cells in PBMCs, duodenum, ileum and colon.

(A) is a pie chart for α-chain showing pairing of TRAV1-2 with various TRAJ in patient 1 and patient 2. (B) is a pie chart for β-chain showing the various TRBV usage of patient # 1 and patient # 2.

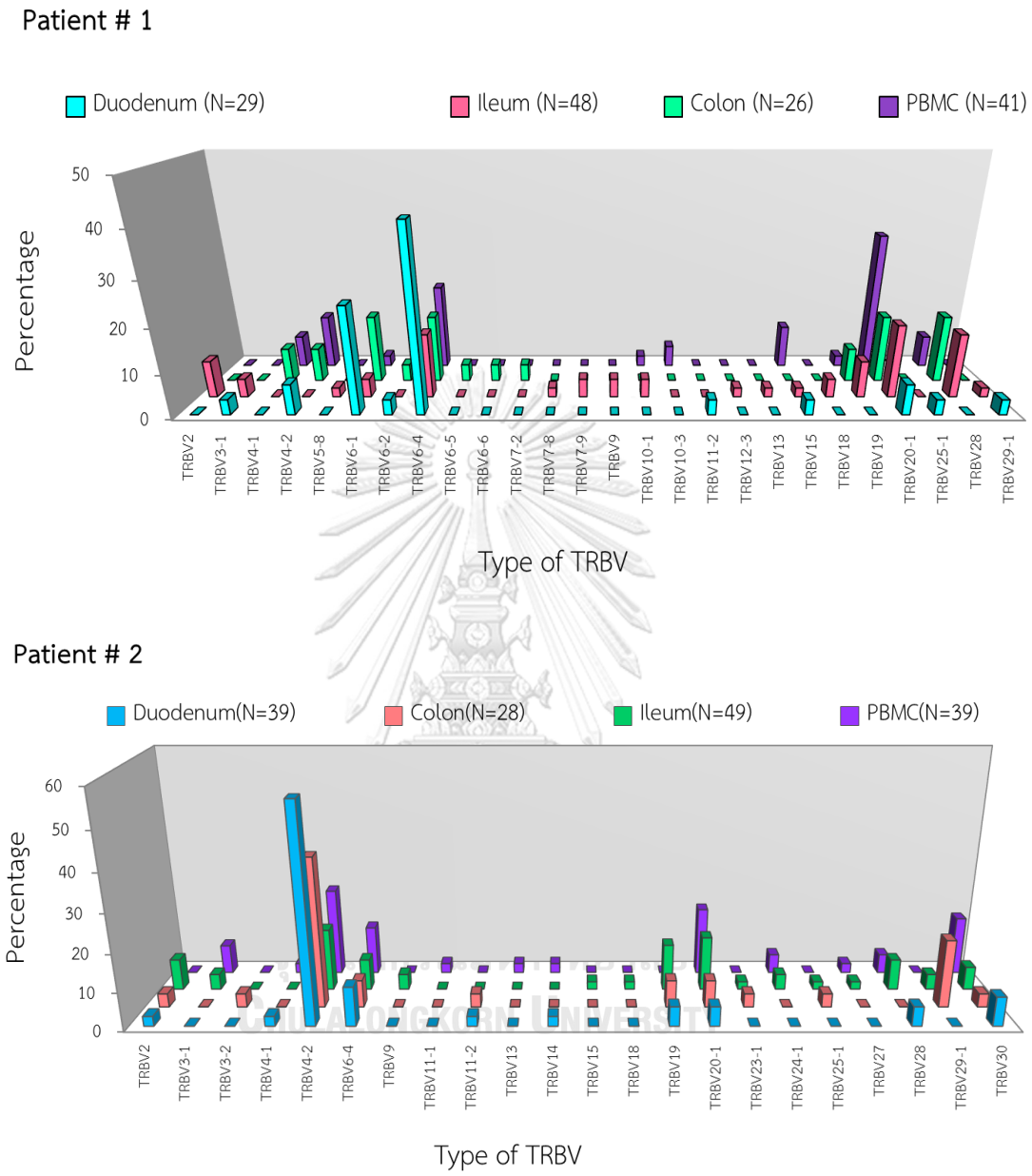


Fig. 12 The comparison graph of type of TRBV founded in different parts of the body.

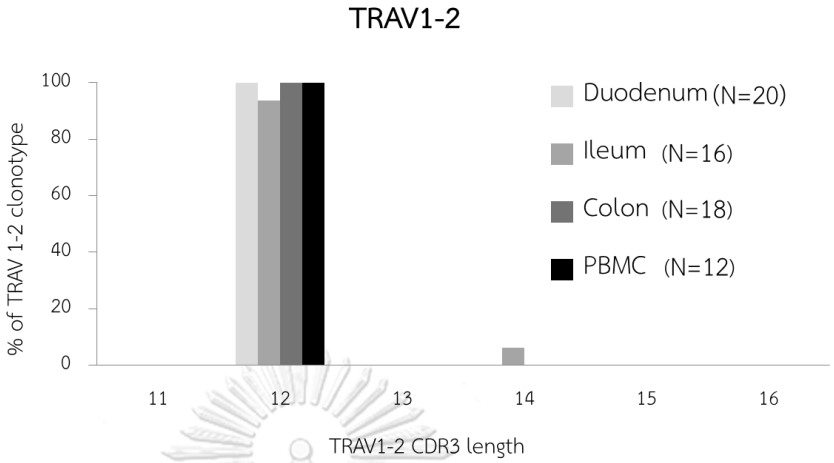
The 3D bar graph show TRBV gene usage in duodenum, ileum, colon and PBMCs of patient # 1 and patient # 2. X-axis was show type of TRBW and Y-axis was show percentage of TRBV in each site.

Length analysis of CDR3 amino acid sequence

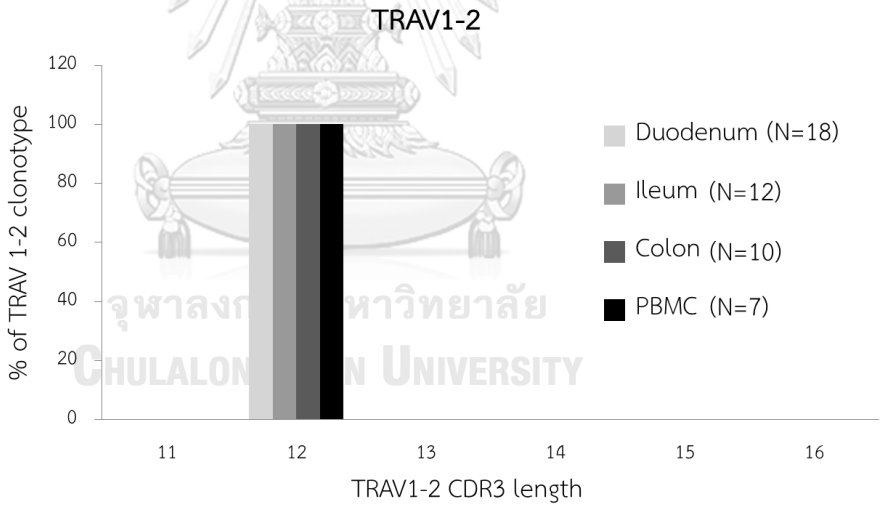
CDR3 is a region for antigen recognition. Moreover, the difference in sequences and the length of CDR3 is according to each T cell clone. The sequence of CDR3 determines the specificity of the TCR, in which one segment of CDR3 is represent represents one T cell clonotype. To evaluate MAIT cells clonality, we measured the CDR3 length of both the α chain and β chain of MAIT cells in the duodenum, ileum, colon and PBMCs of 2 patients. Our results show that the CDR3α length in the duodenum, ileum, colon and PBMC of 2 patients contain 12 amino acid residues. Interestingly, we found one CDR3α that contained 14 amino acid residues in patient # 1 (Fig. 13A). We found that the CDR3β length in duodenum, ileum, colon and PBMCs of both patients contain 11-21 amino acid residues. Moreover, we found that the TRBV CDR3 length of both patients mostly contain 15 amino acid residues followed by 14, 16 and 13 amino acid residues (fig. 13B).

A

Patient # 1



Patient # 2



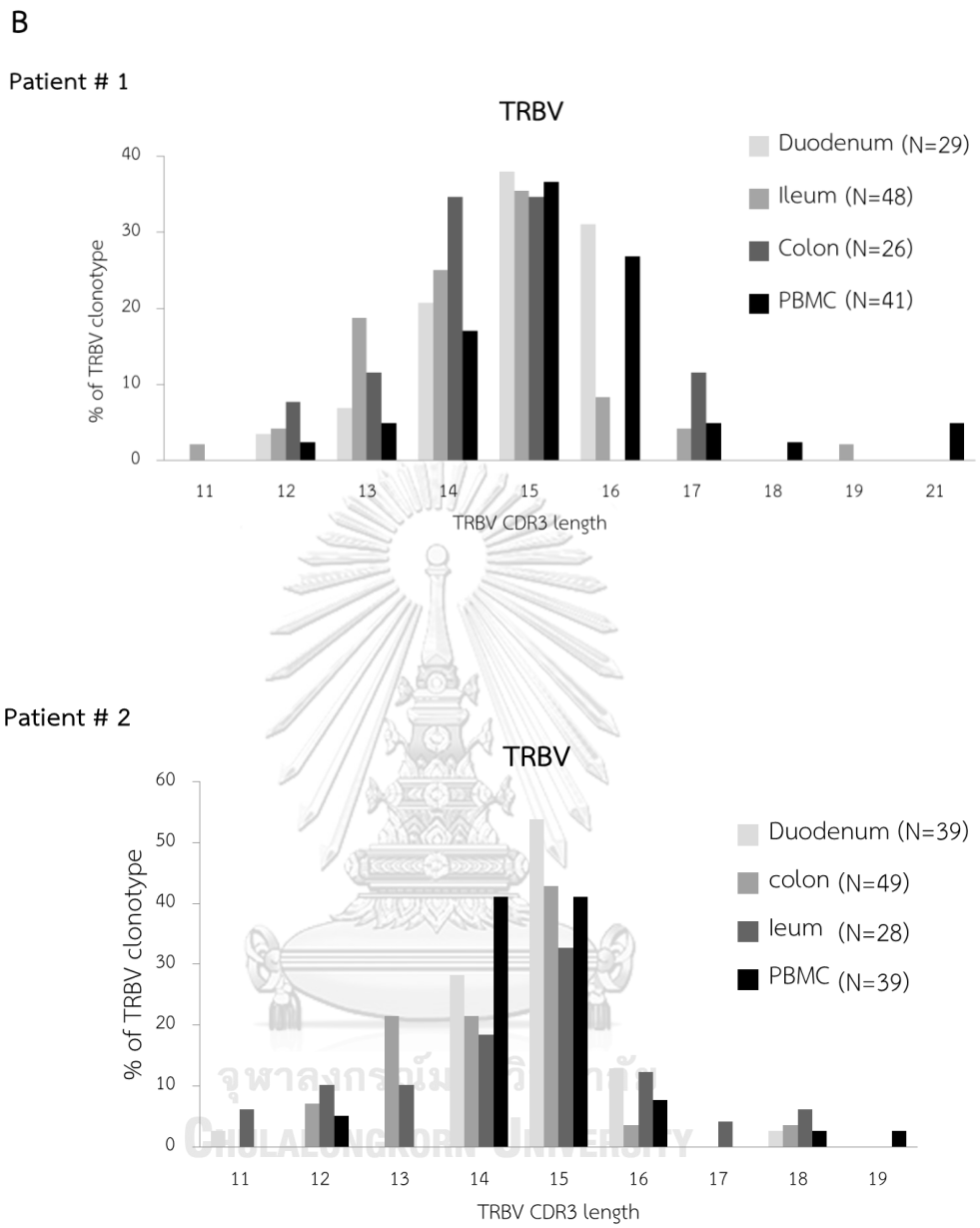


Fig. 13 The bar graph of CDR3 length of MAIT cells in the duodenum ileum colon and PBMCs. (A) The CDR3α length of patient # 1 and patient # 2. (B) The CDR3β length of patient # 1 and patient # 2

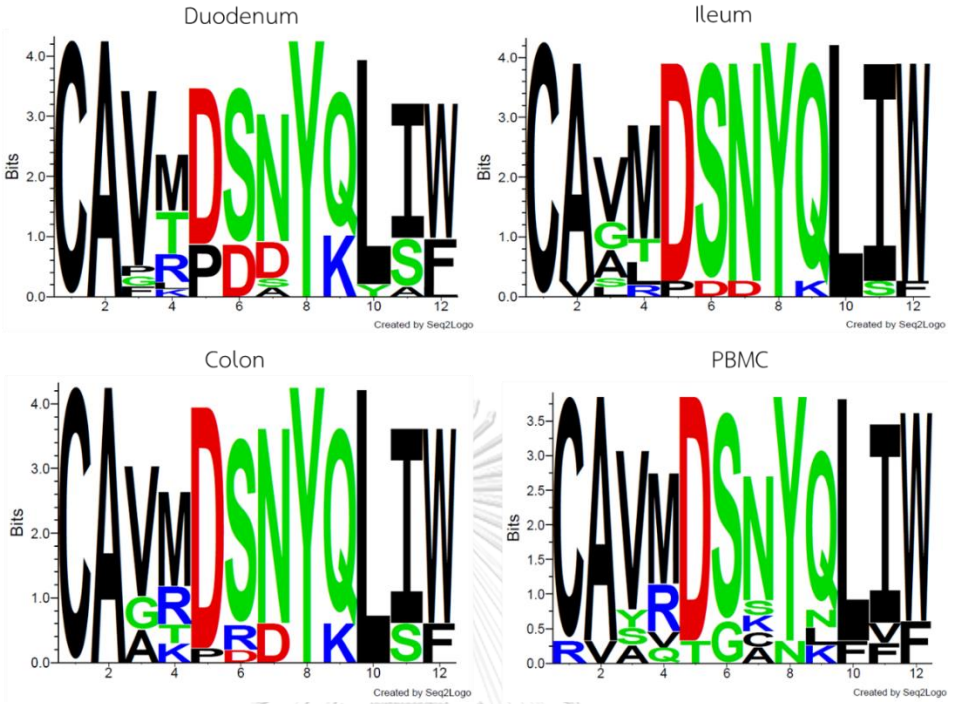
Motif analysis of CDR3 amino acid sequence

CDR3 Motif analysis is represent as fixed-length patterns of CDR3 sequence for investigate the specificity of CDR3, which observe in TCR repertoire. CDR3 motif can show CDR3 sequence that compose of amino acid encoded from germline or non-germline (conserve or not). To evaluate CDR3 amino acid sequence of MAIT cells, we analyzed on http://www.cbs.dtu.dk/biotools/ Seq2Logo. Our results show that CDR3α in duodenum, ileum, colon and PBMCs of both patients contained 12 amino acid residues. The pattern of CDR3α sequence of both patient mostly found CAXXDSNYQLIW. However, CDR3α sequence of both patients were difference some position. For example, in duodenum of patient # 1 is CAXXD(P)S(D)N(X)YQ(K)L(Y)I(S,A)W(F), whereas patient # 2 was CAXXDS(R)N(X)YQ(K)LI(S,M)W(F), in ileum of patient # 1 was CAXXD(P)S(D)N(D)YQLIW, whereas patient # 2 was CAV(T)XDSN(S)YQLIW e.g.(Figure 14A) So, our result suggest that CDR3α sequence of MAIT cells was fixed at some position according to germline encoded and varies at some position according to non-germline encoded (Figure 14A).

Representative logo of amino acid of CDR3β in duodenum, ileum, colon and PBMCs of both patient show that CDR3β contained 11-21 amino acid residues and more diverse than CDR3α. Moreover we found that CDR3β in ileum of both patients was the most diverse. The pattern of CDR3β mostly found CASSXXXXXXXXQYF. So, our results show that CDR3β sequence contained both fixed and varies amino acid residues suggesting that CDR3β compose of germline encoded and non-germline encoded amino acid. In addition, we found that CDR3β sequence of both patients contained glycine (G) and glutamic acid (E). (Figure 14B).

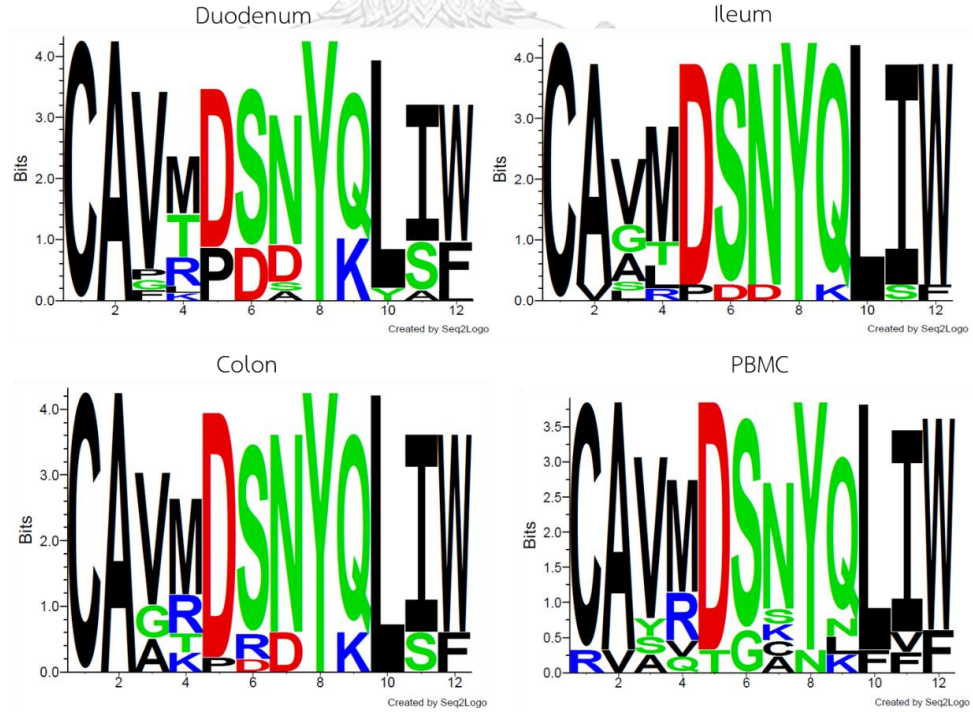
A

Patient # 1 α-chain



A

Patient # 1 α-chain



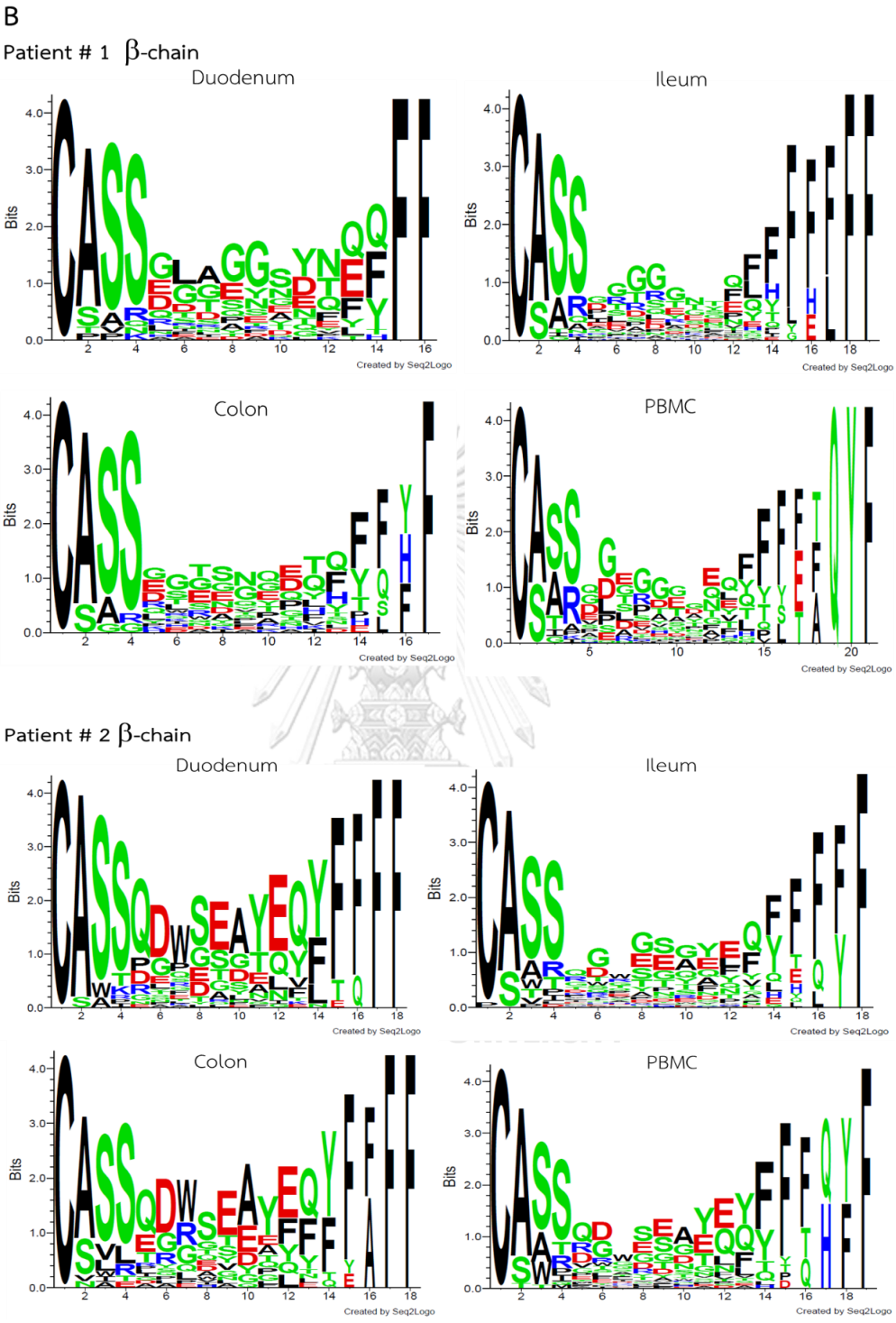


Fig. 14 The graph of amino acid motifs of MAIT cells in duodenum ileum colon and PBMCs. (A) The CDR3α amino acid motifs of patient # 1 and patient # 2. (B) The CDR3β amino acid motifs of patient # 1 and patient # 2.

Chapter V

Discussion

MAIT cells are a subset of T cells [110] that express $\alpha\beta$ TCRs express, surface marker such as CD3, CD8/CD4, CD28 and activation similar to T cells [111]. MAIT cells play a role in the infection and inflammation via cytokine expression such as IFN-γ, TNF-α, IL-17, IL-22, perforin and granzyme B [81]. MAIT cells are abundant in peripheral blood making up to 1-10% and found in gastrointestinal tract, liver, kidney, lymphoid organs, skin, etc [78].

We interested to study MAIT TCR repertoire in gastrointestinal tract to understand the nature of mucosal MAIT TCRs compare with peripheral MAIT TCRs. Due to the basic knowledge of mucosal MAIT TCRs was lacking.

We proliferated MAIT cells using anti-CD3/anti-CD28 beads in the presence of IL-2. Our results suggest that MAIT cells can proliferate *in vitro* by ant-CD3/anti-CD28 beads combined with IL-2. Moreover, before and after proliferation MAIT cells are mostly $CD8^+$ subpopulation followed by double negative and $CD4^+$ subpopulation, which similar to previous research [79]. Moreover, the percentage of $CD8^+$, double negative and $CD4^+$ subpopulation of before proliferation was similar to after proliferation, suggesting that this proliferation was successful and not bias. A polyclonal expansion was regard for TCR repertoire determination due to its impact on TCR repertoire sequence analysis. However, the different TCRs of MAIT cells are not yet know to behave differently under a mitogenic-stimulation. Another limitation of our approach is studying TCR repertoire sequence by PCR. There for, outnumbered for a certain MAIT cells will have a better chance level of bias seen of being amplified.

Moreover, our results found that the clones of β chain was more numerous than α chain due to amplification limitation, leading to the fewer number of α chains was than β chains. Thus, we need to optimize α chain amplification condition and perform more analysis of α chain.

We studied TCR repertoire of MAIT cells in peripheral blood and gastrointestinal tract. The sequence of MAIT TCRs was analyzed on http://www.imgt.org and CDR3 amino acid sequence was created on http://www.cbs.dtu.dk/biotools/ Seq2Logo. Our results showed that TRAJ33 was predominant in the duodenum, ileum, colon and PBMCs of both patients followed by TRAJ20 and TRAJ12. Moreover, in patient # 1, we found TRAV30 and TRAV36 usage but not in patient # 2. Because of TRAJ33, TRAJ20 and TRAJ12 encoded Tyr95, which is conserved in MAIT TCR structure and necessary for interaction with vitamin B metabolites [112]. So, it was not surprising to see TRAJ33, TRAJ20 and TRAJ12 in our result. However, the CDR3α sequence of patient #1 show that TRAJ30 in the ileum is CAVRDMNRDDKIIF and TRAJ36 in the PBMCs is CAAQTGANNLFF. Both sequences show that position 95 was not encoded Tyr95, but encoded for Arg (R) and Asn (N) respectively, suggesting that this two genes are associate with alternative mode of TCRα engagement and consistent with bacterial selectivity [112]. Gold et al., 2014 found that TRAV1-2/TRAJ9 and TRAV1-2/TRAJ39 gene encode TCR were response to *C. albicans* and both genes were not encode Tyr95, while TRAV1-2/TRAJ33, which encode Tyr95 broader response to *M. semegmatis*, *S. thyphimurium* and *C. albicans* suggesting that not only Tyr95 can interaction with antigen and it possible that non-encoding Tyr95 MAIT cells can response to antigen by such unique TCRs.

We demonstrated TRBV usage in 2 patient. Our results show that TRBV6-4 and TRBV20-1 was predominant in the duodenum, ileum, colon and PBMCs of both patients, suggesting that MAIT cells prefers to select certain TRBV family. However, we found different TRBV usage between patient # 1 and patient # 2. TRBV6-1 was predominant in patient # 1, while TRBV4-1 and TRBV30 were predominant in patient # 2

In addition, our results show that the TRBV in the ileum of both patients is most diverse. Moreover, we found that there were overlapping and non-overlapping TRBV gene usage in each site of both patients. For example, TRBV7-9 was found only in the ileum of patient # 1, TRBV9 was found only in the ileum of patient #2. Nevertheless, there were same and different distribution of TRBV gene usage between patient # 1 and patient # 2. For example, TRBV3-1 was found in the duodenum of patient # 1, the PBMCs of patient # 2, and the ileum of both patients; TRAV28 was found in the colon and PBMC of patient # 1, the duodenum and PBMCs of patient two and the ileum of both patient. We hypothesized that the different observation seen may be associated with bacterial selection in the gut. Due to the ligands produced from microbiota as discussed in the table below *[77]*.

Ligand	Microbiota
Vitamin B2	*Lactobacillus lactis*
	Lactobacillus reuteri,
	Lactobacillus fermentum
	Bacteroides fragilis,
	Bifidobacterium adolescentis
	clostridium difficile
	Enterococcus hirae
Vitamin B9	*Lactobacillus pantarum*
	Bifidobacterium adolescentis
	Bifidobacterium dentium
	Streptococcus thermophilus

So, the similarities and difference of ligands in each site may be associated with MAIT TCR gene usage. Moreover, mucosal MAIT cells was more diverse than peripheral MAIT cells, perhaps due to mucosal MAIT usually response to bacteria in gut whereas peripheral blood is sterile site.

Moreover, our result found that TRBV usage in ileum was the most diverse, although the microbiota in the colon was the most diverse. We believe that species of microbiota may not determine diversity of TRBV usage, but this diversity of TRBV usage may be associate with different ligand produced from the microbiota.

Lastly, our result show that CDR3β sequence of both patients contained glycine (G) and glutamic acid (E) at many position. Due to these amino acid has abilities to fine-tune MAIT TCR recognition such as the movement of Gly97 and Glu99 help itself enable contact to ligand and reorientation of Gly98 to from H-bond with Trp69 of MR1 result in remodeling of CDR3β. So, CDR3β flexibility play an important role in the energetic of MAIT TCR-MR1-ligand interaction [113, 114].

In summary, our study obtained new insights on of MAIT TCR repertoire and CDR3 region of MAIT cells in the duodenum, ileum, colon and PBMCs. We hope this knowledge will help fulfill lacking data. However, we should develop how to obtain more clones of α chain in the future. Moreover, we found the interesting TRBV gene usage pattern and distribution, it may be associated with different ligand produced by microbiota, but it is still yet unclear.

REFERENCES

1. Liao, D.H., J.B. Zhao, and H. Gregersen, *Gastrointestinal tract modelling in health and disease.* World J Gastroenterol, 2009. **15**(2): p. 169-76.
2. Bellmann, S., et al., *Mammalian gastrointestinal tract parameters modulating the integrity, surface properties, and absorption of food-relevant nanomaterials.* Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2015. **7**(5): p. 609-22.
3. Mason, K.L., et al., *Overview of gut immunology.* Adv Exp Med Biol, 2008. **635**: p. 1-14.
4. Ramanan, D. and K. Cadwell, *Intrinsic Defense Mechanisms of the Intestinal Epithelium.* Cell Host Microbe, 2016. **19**(4): p. 434-41.
5. Groschwitz, K.R. and S.P. Hogan, *Intestinal barrier function: molecular regulation and disease pathogenesis.* J Allergy Clin Immunol, 2009. **124**(1): p. 3-20; quiz 21-2.
6. Chelakkot, C., J. Ghim, and S.H. Ryu, *Mechanisms regulating intestinal barrier integrity and its pathological implications.* Exp Mol Med, 2018. **50**(8): p. 103.
7. Dhanisha, S.S., et al., *Mucins: Structural diversity, biosynthesis, its role in pathogenesis and as possible therapeutic targets.* Crit Rev Oncol Hematol, 2018. **122**: p. 98-122.
8. Pinzon Martin, S., P.H. Seeberger, and D. Varon Silva, *Mucins and Pathogenic Mucin-Like Molecules Are Immunomodulators During Infection and Targets for Diagnostics and Vaccines.* Front Chem, 2019. **7**: p. 710.
9. Wagner, C.E., K.M. Wheeler, and K. Ribbeck, *Mucins and Their Role in Shaping the Functions of Mucus Barriers.* Annu Rev Cell Dev Biol, 2018. **34**: p. 189-215.
10. Van der Sluis, M., et al., *Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection.* Gastroenterology, 2006. **131**(1): p. 117-29.
11. Ahl, D., et al., *Lactobacillus reuteri increases mucus thickness and ameliorates dextran sulphate sodium-induced colitis in mice.* Acta Physiol (Oxf), 2016.

217(4): p. 300-10.

12. Schroeder, B.O., *Fight them or feed them: how the intestinal mucus layer manages the gut microbiota.* Gastroenterol Rep (Oxf), 2019. **7**(1): p. 3-12.
13. Muniz, L.R., C. Knosp, and G. Yeretssian, *Intestinal antimicrobial peptides during homeostasis, infection, and disease.* Front Immunol, 2012. **3**: p. 310.
14. Lei, J., et al., *The antimicrobial peptides and their potential clinical applications.* Am J Transl Res, 2019. **11**(7): p. 3919-3931.
15. Takahashi, D., et al., *Structural determinants of host defense peptides for antimicrobial activity and target cell selectivity.* Biochimie, 2010. **92**(9): p. 1236-41.
16. Starling, S., *Innate immunity: A new way out for lysozyme.* Nat Rev Immunol, 2017. **17**(9): p. 532.
17. Ragland, S.A. and A.K. Criss, *From bacterial killing to immune modulation: Recent insights into the functions of lysozyme.* PLoS Pathog, 2017. **13**(9): p. e1006512.
18. Niess, J.H. and H.C. Reinecker, *Lamina propria dendritic cells in the physiology and pathology of the gastrointestinal tract.* Curr Opin Gastroenterol, 2005. **21**(6): p. 687-91.
19. Stagg, A.J., *Intestinal Dendritic Cells in Health and Gut Inflammation.* Front Immunol, 2018. **9**: p. 2883.
20. Lombardi, V.C. and S.F. Khaiboullina, *Plasmacytoid dendritic cells of the gut: relevance to immunity and pathology.* Clin Immunol, 2014. **153**(1): p. 165-77.
21. Schridde, A., et al., *Tissue-specific differentiation of colonic macrophages requires TGFbeta receptor-mediated signaling.* Mucosal Immunol, 2017. **10**(6): p. 1387-1399.
22. Atri, C., F.Z. Guerfali, and D. Laouini, *Role of Human Macrophage Polarization in Inflammation during Infectious Diseases.* Int J Mol Sci, 2018. **19**(6).
23. Viola, M.F. and G. Boeckxstaens, *Intestinal resident macrophages: Multitaskers of the gut.* Neurogastroenterol Motil, 2020: p. e13843.
24. Cipriani, G., et al., *Intrinsic Gastrointestinal Macrophages: Their Phenotype and Role in Gastrointestinal Motility.* Cell Mol Gastroenterol Hepatol, 2016. **2**(2): p.

120-130 e1.

25. Zook, E.C. and B.L. Kee, *Development of innate lymphoid cells.* Nat Immunol, 2016. **17**(7): p. 775-82.
26. Eberl, G., J.P. Di Santo, and E. Vivier, *The brave new world of innate lymphoid cells.* Nat Immunol, 2015. **16**(1): p. 1-5.
27. Montalban-Arques, A., et al., *The Innate Immune System in the Gastrointestinal Tract: Role of Intraepithelial Lymphocytes and Lamina Propria Innate Lymphoid Cells in Intestinal Inflammation.* Inflamm Bowel Dis, 2018. **24**(8): p. 1649-1659.
28. Dale, D.C., et al., *Effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) on neutrophil kinetics and function in normal human volunteers.* Am J Hematol, 1998. **57**(1): p. 7-15.
29. Perez-Lopez, A., et al., *Mucosal immunity to pathogenic intestinal bacteria.* Nat Rev Immunol, 2016. **16**(3): p. 135-48.
30. Aviello, G. and U.G. Knaus, *NADPH oxidases and ROS signaling in the gastrointestinal tract.* Mucosal Immunol, 2018. **11**(4): p. 1011-1023.
31. Hall, C.H.T., E.L. Campbell, and S.P. Colgan, *Neutrophils as Components of Mucosal Homeostasis.* Cellular and Molecular Gastroenterology and Hepatology, 2017. **4**(3): p. 329-337.
32. Smith, P.M. and W.S. Garrett, *The gut microbiota and mucosal T cells.* Front Microbiol, 2011. **2**: p. 111.
33. Ferguson, A., *Intraepithelial lymphocytes of the small intestine.* Gut, 1977. **18**(11): p. 921-37.
34. Guy-Grand, D., et al., *Two gut intraepithelial CD8+ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation.* J Exp Med, 1991. **173**(2): p. 471-81.
35. Leishman, A.J., et al., *Precursors of functional MHC class I- or class II-restricted CD8 alpha alpha(+) T cells are positively selected in the thymus by agonist self-peptides.* Immunity, 2002. **16**(3): p. 355-364.
36. Gangadharan, D., et al., *Identification of pre- and postselection TCRalphabeta+ intraepithelial lymphocyte precursors in the thymus.* Immunity, 2006. **25**(4): p.

631-41.

37. van Wijk, F. and H. Cheroutre, *Mucosal T cells in gut homeostasis and inflammation.* Expert Rev Clin Immunol, 2010. **6**(4): p. 559-66.
38. Spencer, J. and L.M. Sollid, *The human intestinal B-cell response.* Mucosal Immunol, 2016. **9**(5): p. 1113-24.
39. Schulz, O. and O. Pabst, *Antigen sampling in the small intestine.* Trends Immunol, 2013. **34**(4): p. 155-61.
40. Hoh, R.A. and S.D. Boyd, *Gut Mucosal Antibody Responses and Implications for Food Allergy.* Frontiers in Immunology, 2018. **9**.
41. Kunkel, E.J. and E.C. Butcher, *Plasma-cell homing.* Nat Rev Immunol, 2003. **3**(10): p. 822-9.
42. Li, Y., L. Jin, and T. Chen, *The Effects of Secretory IgA in the Mucosal Immune System.* Biomed Res Int, 2020. **2020**: p. 2032057.
43. Mathias, A., et al., *Role of secretory IgA in the mucosal sensing of commensal bacteria.* Gut Microbes, 2014. **5**(6): p. 688-95.
44. Ahluwalia, B., M.K. Magnusson, and L. Ohman, *Mucosal immune system of the gastrointestinal tract: maintaining balance between the good and the bad.* Scand J Gastroenterol, 2017. **52**(11): p. 1185-1193.
45. Dillon, S.M., et al., *Age-related alterations in human gut CD4 T cell phenotype, T helper cell frequencies, and functional responses to enteric bacteria.* J Leukoc Biol, 2020. **107**(1): p. 119-132.
46. Brucklacher-Waldert, V., et al., *Cellular Plasticity of CD4+ T Cells in the Intestine.* Front Immunol, 2014. **5**: p. 488.
47. Bamias, G. and F. Cominelli, *Role of type 2 immunity in intestinal inflammation.* Curr Opin Gastroenterol, 2015. **31**(6): p. 471-6.
48. Omenetti, S., et al., *The Intestine Harbors Functionally Distinct Homeostatic Tissue-Resident and Inflammatory Th17 Cells.* Immunity, 2019. **51**(1): p. 77-89 e6.
49. Bedoya, S.K., et al., *Th17 cells in immunity and autoimmunity.* Clin Dev Immunol, 2013. **2013**: p. 986789.
50. Perusina Lanfranca, M., et al., *Biological and pathological activities of*

interleukin-22. J Mol Med (Berl), 2016. **94**(5): p. 523-34.

51. Wang, L., L. Zhu, and S. Qin, *Gut Microbiota Modulation on Intestinal Mucosal Adaptive Immunity.* J Immunol Res, 2019. **2019**: p. 4735040.
52. Luu, M., U. Steinhoff, and A. Visekruna, *Functional heterogeneity of gut-resident regulatory T cells.* Clin Transl Immunology, 2017. **6**(9): p. e156.
53. Wawrzyniak, M., L. O'Mahony, and M. Akdis, *Role of Regulatory Cells in Oral Tolerance.* Allergy Asthma Immunol Res, 2017. **9**(2): p. 107-115.
54. Konjar, S., et al., *Intestinal Barrier Interactions with Specialized CD8 T Cells.* Front Immunol, 2017. **8**: p. 1281.
55. Casado, R.B., et al., *Resident memory CD8 T cells persist for years in human small intestine.* European Journal of Immunology, 2019. **49**: p. 711-711.
56. Boismenu, R. and W.L. Havran, *Modulation of epithelial cell growth by intraepithelial gamma delta T cells.* Science, 1994. **266**(5188): p. 1253-5.
57. Chen, Y., et al., *Protection of the intestinal mucosa by intraepithelial gamma delta T cells.* Proc Natl Acad Sci U S A, 2002. **99**(22): p. 14338-43.
58. McCarthy, N.E. and M. Eberl, *Human gammadelta T-Cell Control of Mucosal Immunity and Inflammation.* Front Immunol, 2018. **9**: p. 985.
59. Mukherjee, S., et al., *Antibacterial membrane attack by a pore-forming intestinal C-type lectin.* Nature, 2014. **505**(7481): p. 103-7.
60. Fahrer, A.M., et al., *Attributes of gammadelta intraepithelial lymphocytes as suggested by their transcriptional profile.* Proc Natl Acad Sci U S A, 2001. **98**(18): p. 10261-6.
61. Middendorp, S. and E.E. Nieuwenhuis, *NKT cells in mucosal immunity.* Mucosal Immunol, 2009. **2**(5): p. 393-402.
62. Brailey, P.M., M. Lebrusant-Fernandez, and P. Barral, *NKT cells and the regulation of intestinal immunity: a two-way street.* FEBS J, 2020. **287**(9): p. 1686-1699.
63. Olszak, T., et al., *Protective mucosal immunity mediated by epithelial CD1d and IL-10.* Nature, 2014. **509**(7501): p. 497-502.
64. Saez de Guinoa, J., et al., *CD1d-mediated activation of group 3 innate lymphoid cells drives IL-22 production.* EMBO Rep, 2017. **18**(1): p. 39-47.

65. Ivanova, D., et al., *NK cells in mucosal defense against infection.* Biomed Res Int, 2014. **2014**: p. 413982.
66. Le Bourhis, L., et al., *Mucosal-associated invariant T cells: unconventional development and function.* Trends Immunol, 2011. **32**(5): p. 212-8.
67. Aron-Wisnewsky, J., J. Dore, and K. Clement, *The importance of the gut microbiota after bariatric surgery.* Nat Rev Gastroenterol Hepatol, 2012. **9**(10): p. 590-8.
68. Clarke, G., et al., *Gut Reactions: Breaking Down Xenobiotic-Microbiome Interactions.* Pharmacol Rev, 2019. **71**(2): p. 198-224.
69. Karigar, C.S. and S.S. Rao, *Role of microbial enzymes in the bioremediation of pollutants: a review.* Enzyme Res, 2011. **2011**: p. 805187.
70. Li, Z., et al., *Effects of Metabolites Derived From Gut Microbiota and Hosts on Pathogens.* Front Cell Infect Microbiol, 2018. **8**: p. 314.
71. Cash, H.L., et al., *Symbiotic bacteria direct expression of an intestinal bactericidal lectin.* Science, 2006. **313**(5790): p. 1126-30.
72. Salzman, N.H., et al., *Enteric defensins are essential regulators of intestinal microbial ecology.* Nat Immunol, 2010. **11**(1): p. 76-83.
73. Salameh, M., et al., *The role of gut microbiota in atopic asthma and allergy, implications in the understanding of disease pathogenesis.* Scand J Immunol, 2020. **91**(3): p. e12855.
74. Lazar, V., et al., *Aspects of Gut Microbiota and Immune System Interactions in Infectious Diseases, Immunopathology, and Cancer.* Front Immunol, 2018. **9**: p. 1830.
75. Shi, N., et al., *Interaction between the gut microbiome and mucosal immune system.* Military Medical Research, 2017. **4**.
76. Yang, H. and Z.J. Duan, *The Local Defender and Functional Mediator: Gut Microbiome.* Digestion, 2018. **97**(2): p. 137-145.
77. Yoshii, K., et al., *Metabolism of Dietary and Microbial Vitamin B Family in the Regulation of Host Immunity.* Front Nutr, 2019. **6**: p. 48.
78. Reantragoon, R., et al., *Mucosal-associated invariant T cells in clinical diseases.* Asian Pac J Allergy Immunol, 2016. **34**(1): p. 3-10.

79. Sugimoto, C., H. Fujita, and H. Wakao, *Mucosal-associated invariant T cells from induced pluripotent stem cells: A novel approach for modeling human diseases.* World J Stem Cells, 2016. **8**(4): p. 158-69.

80. Le Bourhis, L., Y.K. Mburu, and O. Lantz, *MAIT cells, surveyors of a new class of antigen: development and functions.* Curr Opin Immunol, 2013. **25**(2): p. 174-80.

81. Provine, N.M. and P. Klenerman, *MAIT Cells in Health and Disease.* Annu Rev Immunol, 2020. **38**: p. 203-228.

82. Gherardin, N.A., et al., *Diversity of T Cells Restricted by the MHC Class I-Related Molecule MR1 Facilitates Differential Antigen Recognition.* Immunity, 2016. **44**(1): p. 32-45.

83. Gherardin, N.A., et al., *The Diverse Family of MR1-Restricted T Cells.* J Immunol, 2018. **201**(10): p. 2862-2871.

84. Krovi, S.H. and L. Gapin, *Structure and function of the non-classical major histocompatibility complex molecule MR1.* Immunogenetics, 2016. **68**(8): p. 549-59.

85. Kjer-Nielsen, L., et al., *MR1 presents microbial vitamin B metabolites to MAIT cells.* Nature, 2012. **491**(7426): p. 717-23.

86. Birkinshaw, R.W., et al., *MAITs, MR1 and vitamin B metabolites.* Curr Opin Immunol, 2014. **26**: p. 7-13.

87. Keller, A.N., et al., *Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells.* Nat Immunol, 2017. **18**(4): p. 402-411.

88. Wong, E.B., T. Ndung'u, and V.O. Kasprowicz, *The role of mucosal-associated invariant T cells in infectious diseases.* Immunology, 2017. **150**(1): p. 45-54.

89. Xiao, X. and J. Cai, *Mucosal-Associated Invariant T Cells: New Insights into Antigen Recognition and Activation.* Front Immunol, 2017. **8**: p. 1540.

90. Suliman, S., et al., *MR1-Independent Activation of Human Mucosal-Associated Invariant T Cells by Mycobacteria.* Journal of Immunology, 2019. **203**(11): p. 2917-2927.

91. Howson, L.J., M. Salio, and V. Cerundolo, *MR1-Restricted Mucosal-Associated Invariant T Cells and Their Activation during Infectious Diseases.* Front Immunol, 2015. **6**: p. 303.

92. Booth, J.S., et al., *Mucosal-Associated Invariant T Cells in the Human Gastric Mucosa and Blood: Role in Helicobacter pylori Infection.* Front Immunol, 2015. **6**: p. 466.

93. Ussher, J.E., P. Klenerman, and C.B. Willberg, *Mucosal-associated invariant T-cells: new players in anti-bacterial immunity.* Front Immunol, 2014. **5**: p. 450.

94. Magalhaes, I., M. Solders, and H. Kaipe, *MAIT Cells in Health and Disease.* Methods Mol Biol, 2020. **2098**: p. 3-21.

95. Chiba, A., G. Murayama, and S. Miyake, *Mucosal-Associated Invariant T Cells in Autoimmune Diseases.* Front Immunol, 2018. **9**: p. 1333.

96. Laydon, D.J., C.R. Bangham, and B. Asquith, *Estimating T-cell repertoire diversity: limitations of classical estimators and a new approach.* Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1675).

97. Roth, D.B., *V(D)J Recombination: Mechanism, Errors, and Fidelity.* Microbiol Spectr, 2014. **2**(6).

98. Wang, C.Y., et al., *Analysis of the CDR3 length repertoire and the diversity of T cell receptor alpha and beta chains in swine CD4+ and CD8+ T lymphocytes.* Mol Med Rep, 2017. **16**(1): p. 75-86.

99. Kou, Z.C., et al., *T-Cell receptor Vbeta repertoire CDR3 length diversity differs within CD45RA and CD45RO T-cell subsets in healthy and human immunodeficiency virus-infected children.* Clin Diagn Lab Immunol, 2000. **7**(6): p. 953-9.

100. Maru, Y., et al., *Analysis of T cell receptor variable regions and complementarity determining region 3 of infiltrating T lymphocytes in the liver of patients with chronic type B hepatitis.* Intervirology, 2003. **46**(5): p. 277-88.

101. Seach, N., et al., *Double-positive thymocytes select mucosal-associated invariant T cells.* J Immunol, 2013. **191**(12): p. 6002-9.

102. Pellicci, D.G., H.F. Koay, and S.P. Berzins, *Thymic development of unconventional T cells: how NKT cells, MAIT cells and gammadelta T cells emerge.* Nat Rev Immunol, 2020.

103. Huang, S., et al., *Evidence for MR1 antigen presentation to mucosal-associated invariant T cells.* J Biol Chem, 2005. **280**(22): p. 21183-93.

104. Garner, L.C., P. Klenerman, and N.M. Provine, *Insights Into Mucosal-Associated Invariant T Cell Biology From Studies of Invariant Natural Killer T Cells.* Front Immunol, 2018. **9**: p. 1478.

105. Legoux, F., et al., *Microbial metabolites control the thymic development of mucosal-associated invariant T cells.* Science, 2019. **366**(6464): p. 494-499.

106. Stritesky, G.L., S.C. Jameson, and K.A. Hogquist, *Selection of self-reactive T cells in the thymus.* Annu Rev Immunol, 2012. **30**: p. 95-114.

107. Koay, H.F., D.I. Godfrey, and D.G. Pellicci, *Development of mucosal-associated invariant T cells.* Immunol Cell Biol, 2018. **96**(6): p. 598-606.

108. Lamichhane, R., et al., *TCR- or Cytokine-Activated CD8(+) Mucosal-Associated Invariant T Cells Are Rapid Polyfunctional Effectors That Can Coordinate Immune Responses.* Cell Rep, 2019. **28**(12): p. 3061-3076 e5.

109. Shey, M.S., et al., *Contribution of APCs to mucosal-associated invariant T cell activation in infectious disease and cancer.* Innate Immun, 2018. **24**(4): p. 192-202.

110. Held, K., et al., *T-cell receptor repertoire of human peripheral CD161hiTRAV1-2+ MAIT cells revealed by next generation sequencing and single cell analysis.* Hum Immunol, 2015. **76**(9): p. 607-14.

111. Godfrey, D.I., et al., *The biology and functional importance of MAIT cells.* Nat Immunol, 2019. **20**(9): p. 1110-1128.

112. Gold, M.C., et al., *MR1-restricted MAIT cells display ligand discrimination and pathogen selectivity through distinct T cell receptor usage.* J Exp Med, 2014. **211**(8): p. 1601-10.

113. Lopez-Sagaseta, J., et al., *MAIT Recognition of a Stimulatory Bacterial Antigen Bound to MR1.* Journal of Immunology, 2013. **191**(10): p. 5268-5277.

114. Eckle, S.B., et al., *A molecular basis underpinning the T cell receptor heterogeneity of mucosal-associated invariant T cells.* J Exp Med, 2014. **211**(8): p. 1585-600.

APPENDIX

APPENDIX A

REAGENTS PREPARATION

1. Phosphate Buffer Saline (PBS)

   Stock solution (10x)

NaCl	80	g.
Na_2HPO_4	11.5	g.
KCl	2	g.
KH_2PO_4	2	g.
ddH_2O	1000	ml.

   Working solution (1X)

PBS 10x	100	ml
ddH_2O	900	ml.

2. Supplemental Complement (SC)

RPMI		320	ml.
Non-essential Amino Acid Solution		100	ml.
Penicillin Streptomycin Solution		80	ml.
HEPEES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid		11.9	ml.
L-glutamine	100	ml./3	g.
b-mercaptoethanol		35	μl

   Filtered by 0.2 μm and aliquod to 30 ml., store at -20 °C

3. Fetal bovine Serum (FBS)

   Heat-inactivated at 56 °C for 30 min, store at -20 °C

4. RF 10 Medium

10 % FBS	60	ml.
SC	30	ml.

RPMI	500	ml.

5. Digestion buffer

PBS	1960	µl
FCS	40	µl
Collagenase type IV in $CaCl_2$	200	µl

6. Freezing Reagent

FBS	30	ml.
Dimethyl sulfoxide (DMSO)	3.3	ml.

7. FACS Buffer

PBS	100	ml.
FBS	2	ml

8. TAE buffer

Stock solution (50X)

Tris Base (MW=121.1)	242	g.
Glacial Acetic Acid	57.1	ml
0.5 M EDTA	100	ml

Mix Tris with stir bar to dissolve in 600 ml of ddH_2O.

Add the EDTA and Acetic Acid

Bring final volume to 1000 ml. with ddH_2O

Working solution

TAE buffer	20	ml.
ddH_2O	980	ml.

APPENDIX B

CHEMICAL AND REAGENTS

1.	Phosphate Buffer Saline (PBS)	THAILAND
2.	Roswell Park Memorial Institute (RPMI) medium (Gibco)	USA
3.	Ficoll-paque (GE Healthcare)	SWEEDEN
4.	Collagenase IV	THAILAND
5.	Fetal bovine Serum	THAILAND
6.	Dimethyl sulfoxide (DMSO) (AMRESCO)	USA
7.	Anti-human CD3-PE antibody	USA
8.	Anti-human CD3-PE/Cy7 antibody	USA
9.	Anti-human CD3-FITC antibody	USA
10.	Anti-human CD8-APC antibody	USA
11.	Anti-human CD161-APC antibody	USA
12.	Anti-human Vα7.2-FITC antibody	USA
13.	5x VILO (Invitrogen)	USA
14.	10x Superscript (Invitrogen)	USA
15.	Triton X-100	USA
16.	DNase RNase free water (Invitrogen)	USA
17.	10x PCR buffer (Invitrogen)	USA
18.	50 mM $MgCl_2$ (Invitrogen)	USA
19.	dNTP (Invitrogen)	USA
20.	TRAV external (IDT)	USA
21.	TRAC external (IDT)	USA
22.	TRBV internal (IDT)	USA
23.	TRBC internal (IDT)	USA

24. Taq DNA (Invitrogen)	USA
25. Agarose	USA
26. SYBR Safe (Invitrogen)	USA

APPENDIC C

MATERIAL AND EQUIPMENT

1. Flow cytometer (BD LSR II, BD FACSAria II)
2. Hemacytometer
3. Falcon Tube 15 and 50 ml.
4. Disposable plastic transfer pipettes
5. Glass pipettes 2, 5, 10 and 25 inch
6. Shacker
7. Water bath
8. 96 U-bottom plate
9. 96 V-bottom plate
10. 96-well semi-skirt PCR plate
11. Multichannel pipetted 1-10 and 5-50 μl
12. Autopipette 10, 20, 50, 100, 200 and 1000 μl
13. SimpliAmp Thermal Cycler, Thermo Fisher
14. Image Quant Las 500, (GE Healthcare)

VITA

NAME	Thidarat Kongkaew
DATE OF BIRTH	7 September 1993
PLACE OF BIRTH	Chonburi, Thailand
INSTITUTIONS ATTENDED	2011-2015 B.Sc Microbiology Srinakharinwirot University, Thailand
HOME ADDRESS	48 Moo 8 Tha Ngam, Inburi, Singburi 16110