การแสดงออกและหน้าที่ของโทไล้รีเซ็บเตอร์ บนเดนไดรติกเซลล์ และเซลล์ที่ทำหน้าที่นำเสนอแอนติเจนชนิดต่างๆ ในไพรเมท (ลิง)

นางสาวชุติธร เกตุลอย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EXPRESSION AND FUNCTION OF TOLL-LIKE RECEPTORS ON DENDRITIC CELLS AND OTHER ANTIGEN PRESENTING CELLS FROM NON-HUMAN PRIMATES

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เซลล์ที่ทำหน้าที่นำเสนอแอนติเจน โดยเฉพาะอย่างยิ่ง เดนไดรติกเซลล์ มีบทบาทสำคัญในการตอบสนองทางภูมิคุ้มกันต่อการติดเชื้อโรค โดยผ่านทางโทโล้รีเซ็บเตอร์ ดังนั้นสารที่จับและกระตุ้นอย่างจำเพาะ หรือที่เรียกว่า ไลแกนด์ ของโทโล้รีเซ็บเตอร์ จึงเป็นตัวเลือกที่น่าสนใจสำหรับการนำมาใช้เพื่อเพิ่มความสามารถในการกระตุ้นภูมิคุ้มกันของวัคซีน หรือที่เรียกว่า แอดจูแวน (สารเสริมประสิทธิภาพของวัคซีน) ทั้งในคนและสัตว์ทดลอง อย่างไรก็ตามยังไม่การศึกษาเกี่ยวกับการแสดงออกของโทโล้รีเซ็บเตอร์ในไพรเมท เช่น ในลิง ผู้วิจัยจึงได้ทำการศึกษาเปรียบเทียบข้ามสายพันธุ์ของ การแสดงออกของโทโล้รีเซ็บเตอร์ชนิดที่ 3, 4, 7, 8, และ 9 บนเซลล์ที่ทำหน้าที่นำเสนอแอนติเจนชนิดต่าง ๆ ระหว่าง คน ลิง และหนู ซึ่งพบว่ากลุ่มย่อยต่าง ๆ ของเดนไดรติกเซลล์ในกระแสเลือดของลิงมีการแสดงออกของโทโล้รีเซ็บเตอร์ ชนิดต่าง ๆ เหมือนกับเดนไดรติกเซลล์ของคน แต่มีความแตกต่างจากเดนไดรติกเซลล์ของหนูในบางส่วน ในลิงและคน พบว่าเดนไดรติกเซลล์ชนิดไมอีลอยด์ มีการแสดงของโทโล้รีเซ็บเตอร์ชนิดที่ 3, 4, 7 และ 8 ขณะที่เดนไดรติกเซลล์ชนิดพลาสม่าไซตอยด์ มีการแสดงของโทโล้รีเซ็บเตอร์ชนิดที่ 7 และ 9 เท่านั้น ซึ่งแตกต่างจากหนู เดนไดรติกเซลล์ทั้งสองกลุ่มมีการแสดงของโทโล้รีเซ็บเตอร์ทุกชนิด (3, 4, 7, 8 และ 9) นอกจากนี้ยังพบว่าการแสดงของโทโล้รีเซ็บเตอร์บนเซลล์ที่ทำหน้าที่นำเสนอแอนติเจนชนิดอื่น ๆ ในลิงนั้น เหมือนกับในคน ได้แก่ มีการแสดงออกของโทโล้รีเซ็บเตอร์ชนิดที่ 3, 4, 8 และ 9 บนเดนไดรติกเซลล์ชนิดที่ได้มาจากการเลี้ยงเซลล์โมโนไซต์, มีการแสดงออกของโทโล้รีเซ็บเตอร์ชนิดที่ 4, 7 และ 8 บนเซลล์โมโนไซต์ และมีการแสดงออกของโทโล้รีเซ็บเตอร์ชนิดที่ 4, 7, 8 และ 9 บนบีเซลล์ อย่างไรก็ตามการตอบสนองของเซลล์ที่ทำหน้าที่นำเสนอแอนติเจนชนิดต่าง ๆ ต่อไลแกนด์ของโทโล้รีเซ็บเตอร์ในลิง มีความแตกต่างบางส่วนจากคน ในส่วนของการเปลี่ยนแปลงทางฟีโนไทป์ และการสร้างไซโตไคน์ แต่ที่เห็นเด่นชัดคือ ตรงข้ามกับในคน ในลิงไม่พบการสร้างของไซโตไคน์ ชนิด IL-12p70 จากเดนไดรติกเซลล์ชนิดที่ได้มาจากการเลี้ยงเซลล์โมโนไซต์ เมื่อกระตุ้นด้วยไลแกนด์ของโทโล้รีเซ็บเตอร์ชนิดต่าง ๆ ผลที่ได้จากงานวิจัยในครั้งนี้ แสดงให้เห็นถึงความสำคัญในการเลือกสัตว์ทดลองให้เหมาะสม เพื่อช่วยในกระบวนการทดสอบหาแอดจูแวนจากไลแกนด์ของโทโล้รีเซ็บเตอร์ชนิดต่าง ๆ เพื่อใช้ได้ในคนต่อไป

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต ชุติธร เกตุลอย

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CHUTITORN KETLOY : EXPRESSION AND FUNCTION OF TOLL-LIKE RECEPTORS ON DENDRITIC CELLS AND OTHER ANTIGEN PRESENTING CELLS FROM NON-HUMAN PRIMATE. THESIS ADVISOR: PROF. KIAT RUXRUNGTHAM, THESIS COADVISOR: SATHIT PICHYANGKUL, PH.D., 101pp.

Antigen presenting cells (APCs), especially dendritic cells (DCs), play a crucial role in immune responses against infections by sensing microbial invasion through toll-like receptors (TLRs). Thus, TLR ligands are attractive candidates for use in humans and animal models as vaccine adjuvants. So far, no studies have performed on TLR expression in non-human primates such as rhesus macaques. We therefore performed a comparative cross-species study on TLR expression patterns (TLR3, 4, 7, 8 and 9) of APCs in human, rhesus macaques and mice. We demonstrate that blood DC subsets of rhesus macaque expressed the same sets of TLRs as human DCs but substantially differed from mouse DC subsets. In macaque and human, myeloid DCs (MDCs) expressed TLR3, 4, 7 and 8 whereas plasmacytoid DCs (PDCs) expressed only TLR7 and 9, in contrast to mouse, both DC subsets expressed all TLRs (TLR3, 4, 7, 8 and 9). Additionally, TLR expression patterns in macaque monocyte-derived dendritic cells (mo-DCs) (i.e., TLR3, 4 and 8), monocytes (i.e., TLR4, 7, and 8) and B cells (i.e., TLR4, 7, 8, and 9) were also similar to their human counterparts. However, the responsiveness of macaque APCs to certain TLR ligands partially differed from those of human in terms of phenotype differentiation and cytokine production. Strikingly, in contrast to human mo-DCs, no IL-12p70 production was observed when macaque mo-DCs were stimulated with TLR ligands. Our results provide important information for a rational design of animal models in evaluating TLR ligands as adjuvant *in vivo*.

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Co-Advisor's signature Sathit P

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LIST OF ABBREVIATIONS

APCs	=	antigen-presenting cells
BM	=	bone-marrow
bp	=	base pair
ºC	=	degree celcius
cDC	=	conventional DC
CD	=	cluster of differentiation
CFA	=	complete freunds adjuvant
CM	=	complete medium
CpG ODN	=	CpG oligodeoxynucleotides
CTL	=	cytotoxic T lymphocyte
DCs	=	dendritic cells
ds	=	double stranded
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxynitrogentriphosphates
et al.	=	et alii
FACS	=	fluorescense activated cell sorter
FCS	=	fetal calf serum
Flt3-L	=	fms-related tyrosine kinase 3 ligand
GM-CSF	=	granulocyte-macrophage colony-stimulating factor
HPCs	=	haematopoietic progenitor cells
IFN	=	interferon
Ig	=	immunoglobulin
IL-1Rs	=	interleukin-1 receptors
IL	=	interleukin
IRF	=	interferon-regulatory factor
LCs	=	langerhans cells
LPS	=	lipopolysaccharide
LRR	=	leucine-rich repeat
M-CSF	=	macrophage colony-stimulating factor

MACS	=	immunomagnetic cell sorting
MDCs	=	myeloid dendritic cells
MFI	=	mean fluorescence intensity
$MgCl_2$	=	magnesium chloride
MHC	=	major histocompatibility complex
mL	=	milliliter
mo-DCs	=	monocyte-derived dendritic cells
MyD88	=	myeloid differentiation factor 88
μg	=	microgram
μL	=	microliter
TIR	=	Toll/IL-1R
TNF	=	tumour-necrosis factor
TRIF	=	TIR-domain-containing adaptor protein inducing IFN-β
PAMPs	=	pathogen-associated molecular patterns
PBMCs	=	peripheral blood mononuclear cells
PCR	=	polymerase chain reaction
PDCs	=	plasmacytoid dendritic cells
poly (I:C)	=	polyinosine-polycytidylic acid
PRRs		pattern-recognition receptors
RNA	=	ribonucleic Acid
ss	=	single stranded
Th cell	=	T helper cell
TIR	=	Toll/IL-1 receptor homologous
TLRs	=	toll-like receptors

CHAPTER I

INTRODUCTION

1.1 BACKGROUND AND RATIONALE

In order to develop successful and effective vaccines, vaccine antigens should be aimed to target the antigen presenting cells (APCs), especially dendritic cells (DCs). Moreover, it has become noticed over the last years that the stronger and more effective vaccine immunogenicity could be induced by the combination of the vaccine antigens with so-called “adjuvants” [1]. Nowadays, only an aluminium salt (alum) and squalane oil/water emulsion (MF-59) have been approved by US Food and Drug Administration (FDA) for using as adjuvants in human vaccine [2]. In this regard, a number of researchers try to explore new candidate vaccine adjuvants. Activation via the toll-like receptors (TLRs), which are highly presented on APCs, had attracted attention as a candidate for adjuvant development [3, 4].

TLRs function as pathogen-recognition receptors (PRRs) and recognize conserved pathogens via pathogen-specific molecular patterns (PAMPs) [5]. To date, at least 10 members of TLRs have been identified in both humans and mice [5]. Several ligands for TLRs have been identified, including lipoproteins and peptidoglycans for TLR2, double stranded RNA of viral origin for TLR3, lipopolysaccharide (LPS) from Gram negative bacteria for TLR4, flagellin, a protein found in bacterial flagella, for TLR5, single stranded RNA and synthetic imidazoquinoline compounds for TLR7 and TLR8, and unmethylated CpG motifs found in bacterial DNA for TLR9 [4]. Binding to PAMPs by TLRs lead to the initiation of an intracellular signaling cascade [6] that results in upregulation of cell surface expression of co-stimulatory and major histocompatibity complex (MHC) class II molecules, and secretion of cytokines and chemokines. These events lead to

initiation of adaptive immune responses [3, 4, 7]. Therefore, TLRs are good targets for rational adjuvant development.

APCs are composed of several types of cells such as monocytes/macrophages, B cells, and DC subsets which are divided into plasmacytoid DCs (PDCs) and myeloid DCs (MDCs) [8, 9]. Several studies reported that distinct APCs express different patterns of TLRs. [10-13]. In humans, B cells and PDCs are the only immune cells that are known to express TLR9 and that can be activated by CpG [11], whereas TLR7 and 8 are found on B cells, PDCs and MDCs and on monocytes [10-13]. However, in mice, a commonly used animal model for testing novel adjuvant formulations, there are important differences in the TLR expression patterns on APCs as compared to humans. In mice, TLR9 is broadly expressed on all major DC subtypes (PDCs and MDCs) as well as in B cells, macrophages and monocytes [14, 15]. Moreover, TLR8 does not function in mice [15]. Overall, differential expression of TLRs in distinct DC subsets may lead to the different outcome of adaptive immune responses. These differences affect the validity of mouse models for adjuvant evaluation, making direct extrapolation from mouse data to human difficult.

The limitations of mouse models, especially for their different of TLRs, lead to the exploration of new animal models to test TLR-related vaccine adjuvants. Non-human primates such as rhesus macaques are closely related to humans with more recent work showing that macaques also have circulating MDCs and PDCs as in humans [16-18]. Moreover, several groups have used TLR ligands as adjuvants in macaques, including immunization of the antigen together with TLR3 [19], TLR7/8 [20], or TLR9 ligands [21]. However, so far, it is not known if a similar set of TLRs is present on APCs from human and macaque. Our aim was to establish whether macaque DC subsets and other APCs express TLR patterns similar to human and to study the functionality of the TLRs expressed on macaque APCs. This study provides important information for a rational design of animal models in evaluating TLR ligands as adjuvant *in vivo*.

1.2 RESEARCH QUESTIONS

1.2.1 Whether the TLR mRNA expression patterns (TLR 3, 4, 7, 8, and 9) on DC subsets (MDCs, PDCs, mo-DCs) and other APC (monocytes and B cells) differ among different species: mouse, human and non-human primate.

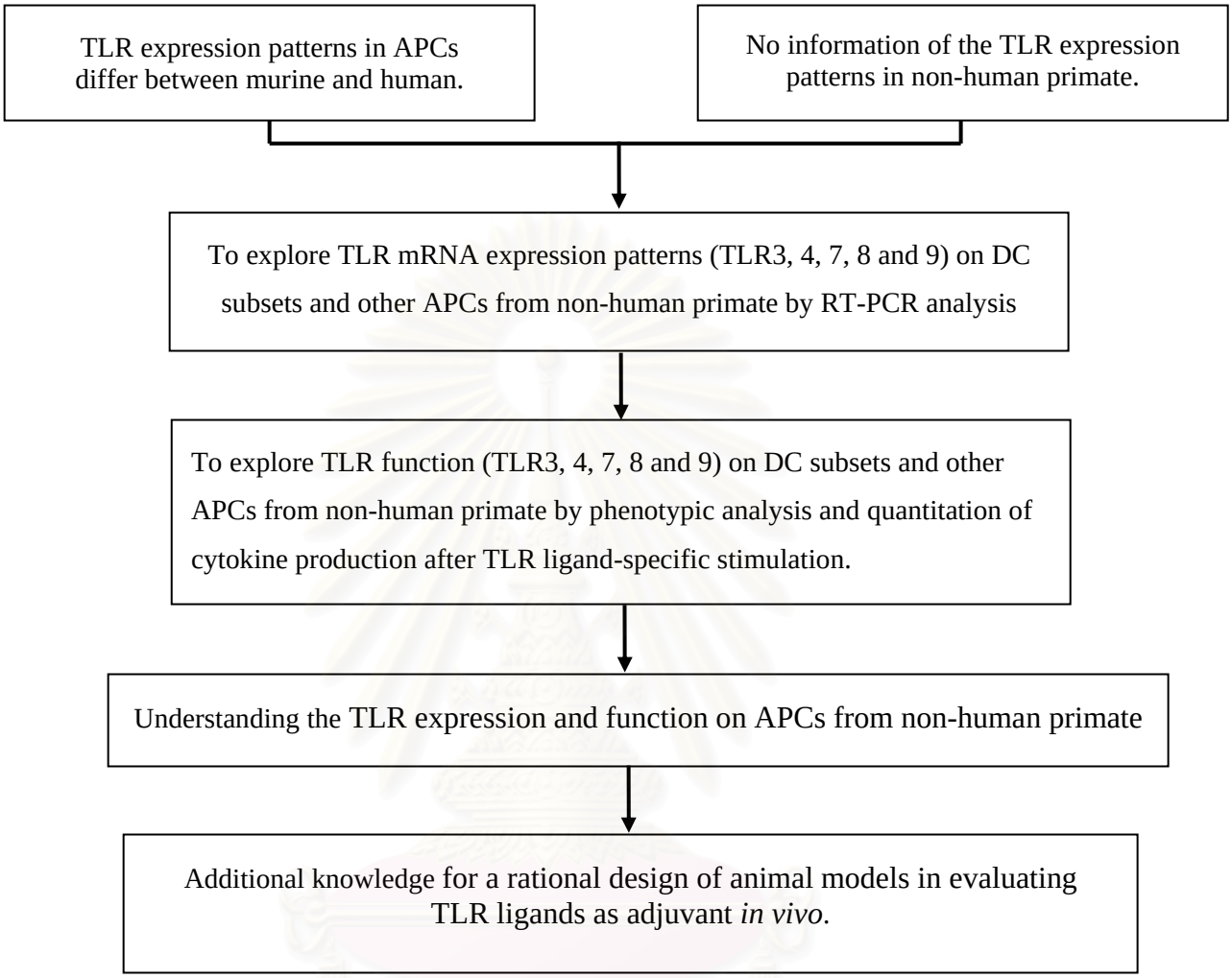
1.2.2 Whether the TLR function (TLR 3, 4, 7, 8, and 9) on DC subsets (MDCs, PDCs, mo-DCs) and other APCs (monocytes and B cells) from non-human primate differ from human.

1.3 OBJECTIVES OF THIS RESEARCH

1.3.1 To compare the TLR mRNA expression patterns (TLR 3, 4, 7, 8, and 9) on DC subsets (MDCs, PDCs, mo-DCs) and other APCs (monocytes and B cells) among different species: mouse, human and non-human primate by RT-PCR analysis.

1.3.2 To explore the TLR function (TLR 3, 4, 7, 8, and 9) on DC subsets (MDCs, PDCs, mo-DCs) and other APCs (monocytes and B cells) from non-human primate by analysis of phenotypic changes (CD40, CD83, or CD86) and cytokine productions (IFN-α, TNF-α, and IL-12p70) after various TLR ligand-specific stimulations.

1.4 CONCEPTUAL FRAMEWORK



1.5 KEY WORDS

- toll-like receptors (TLRs)
- dendritic cells (DCs)
- rhesus macaques
- antigen presenting cells (APCs)

1.6 EXPECTED BENEFIT AND APPLICATION

At present, few adjuvants for use in human vaccine have been approved by US Food and Drug Administration (FDA) [2]. Nowadays, activating the immune system via TLRs is a new target for vaccine adjuvant development. To explore the novel TLR ligands as adjuvants for humans, factors to keep in mind are species-specificity of TLR expression, ligand recognition and DC subsets. Mice can produce a broad immune response when stimulated with TLR ligands especially CpG ODN (TLR9 ligand). However, TLR9 expression may not be present in all DC subsets in primates. The limitations of mouse models to answer all questions surrounding TLRs lead to the exploration of new animal models to test TLR-related vaccine adjuvants. Our study will open new ways in the field of TLR research in non-human primates. Comparative cross-species study of TLR expression and function on DC subsets and APCs in mice, non-human primate and human, will provide additional knowledge of animal models assessment for TLR-related vaccine adjuvants in humans.

CHAPTER II

LITERATURE REVIEW

The immune system is consists of the molecules, cells, tissues, and organs that collectively function to protect the individual against pathogen organisms. It can be broadly classified into adaptive and innate immune systems. Adaptive immunity mediates a delayed specific response and develops immunological memory after encounter with pathogens, whereas innate immunity is a nonspecific immune response that is generated immediately after exposure to pathogens [22, 23] However, recent studies showed that innate immunity is able to discriminate between pathogens and self [24-26]. Moreover, the recently studies showed that innate immunity is able to discriminate between pathogens and self [24-26]. In addition, the activation of innate immunity can also induce acquired immunity. Therefore, the recent vaccine development is focused on not only the activation of acquire immunity but also innate immunity.

Key to the development of effective vaccines is to trigger strong and long-lived immunological specific B and T cell memory to a specific pathogens. **Antigen presenting cells (APCs)**, and especially **dendritic cells (DCs)**, play an important role in the presentation of vaccine antigen to the immune system. Also, it is now well-known that successful vaccines should contain not only a vaccine antigen, but also a good **adjuvant** that efficiently activates the innate immune system for efficient vaccine immunogenicity [1].

Adjuvants are defined as compounds that can enhance or modulate the immunogenicity of a vaccine antigen. Recently studies revealed that the majority of adjuvants activate the immune system via **Toll-like receptor** (TLR) [24, 27] . Therefore, detailed knowledge of TLR expression on APCs and subsequent functional consequences of TLR ligation will be useful for the development of adjuvant formulations.

2.1 ANTIGEN-PRESENTING CELL

An antigen-presenting cell (APC) is a cell that presents the antigens on its surface in associated with major histocompatibility complex (MHC) molecules (peptide-MHC complexes). MHC-peptide can be recognized by T cells expressing a specific T cell receptor leading to the activation of T cells [28]. There are three types of APCs in the immune system.

2.1.1 *Dendritic cells*

Dendritic cells (DCs) are the most potent APCs that can induce both CD8 and CD4 T-cell responses to antigens presented on MHC-I and MHC-II molecules, respectively. They play an important role in manipulating the immune responses for therapeutic effects, for example, in vaccination.

2.1.2 *Macrophages*

Macrophages are APCs that actively phagocytose large particles. Therefore, they play an important role in presenting antigens derived from phagocytosed infectious organisms such as bacteria and parasites. Most macrophages express low levels of MHC-II molecules, and much higher levels are induced by the cytokine interferon-γ (IFN-γ).

2.1.3 *B cells*

B cells are the least efficient APCs that have surface immunoglobulins (specific antigen receptors) and ingest soluble proteins by pinocytosis. Because B cells do not express co-stimulatory molecules, they are not so effective as APCs. APC-capacity of B cells can be boosted through activation by Th cells.

.

Table I : Summary table of Antigen Presenting Cells

	Dendritic Cell	Macrophage	B cell
MHC-II Expression	Always expressed Inducible upon activation	Low levels Induced by bacteria and/or bytokines	Always expressed Inducible upon cctivation
Antigen type and presentation by MHC	Intracellular & extracellular antigens: presentation via MHC-I & II	Extracellular antigens: presentation via MHC-II	Extracellular antigens binds to specific Ig receptors: presentation via MHC-II
Co-Stimulation	Always expressed at high levels Inducible upon activation	Low levels Induced by cacteria and/or cytokines	Low levels Inducible upon cctivation
Location	Lymphoid tissue Connective tissue Epithelium Blood	Lymphoid tissue Connective tissue Body cavities	Lymphoid tissues Blood

2.2 DENDRITIC CELL

DCs were firstly identified as Langerhans cells (LCs) in the skin in 1868, and in 1973 by Ralph M. Steinman and Zanvil A. Cohn [29]. They have a main function in capturing antigens and initiating T cell-mediated immune responses. It is well known that DCs are the most potent APCs, due to the higher expression of MHC molecules, in comparison with B cells, monocytes and macrophages [30]. Furthermore, a small number of DCs can stimulate a large number of T cells [31].

2.2.1 Function of DCs

The function of DCs is associated with their maturation stages. There are three stages of DC maturation : precursor DCs, immature DCs, and mature DCs [8].

2.2.2.1 *Precursor DCs*

The precursor DCs in the blood circulation are derived from the DC progenitors in the bone marrow. Under different condition, such as different cytokine stimulation, precursor DCs grow and differentiate into three distinct phenotypes of mature DCs: interstitial DCs, LCs, and lymphoid DCs. Furthermore, three subsets of precursors have been identified: $CD14^+$ monocytes, the $CD11c^+$ precursor DC, and the $CD11c^-$ precursor DC [32].

2.2.2.2 *Immature DCs*

Immature DCs are located throughout almost every tissue in which they can detect and acquire pathogens (such as bacteria, fungi, viruses) by endocytosis or through specific pattern recognition receptors such as the toll-like receptors (TLRs). Following these conditions, the immature DCs leave the tissues and migrate to the secondary lymphoid organs. During the migration, the DCs become mature resulting in upregulation of MHC and costimulatory molecules and secretion of proinflammatory cytokines [33].

2.2.2.3 *Mature DCs*

When DCs reach the secondary lymphoid organs, such as spleen and lymph nodes, they are considered as mature DCs or *interdigitating DCs* [34]. Here, DCs present the antigen to stimulate both $CD8^+$ cells and $CD4^+$ cells via MHC-I and MHC-II molecules, respectively [35]. Then, lymphocytes become activated and can expand and differentiate. Finally, lymphocytes migrate back to the injured tissue to flight the pathogens.

Table II : Comparison of immature and mature dendritic cells

Peripheral tissues ⟶ Lymphoid organs

Immature Dendritic Cells	Mature Dendritic Cells
No prominent dendrites	Prominent dendrites
High levels of cytoplasmic MHC class II molecules	High level of surface expression of MHC class I, class II molecules
Low levels of costimulatory and adhesion molecules	High levels of costimulatory and adhesion molecules
Phagocytic activity	Poor phagocytic activity
Poor stimulation of T cells	Potent stimulation of T cells

2.2.2 Types of Dendritic Cells

2.2.2.1 *Humans*

2.2.2.1.1 *In vivo*

In human, much of knowledge about DCs has come from the study of DC subsets in the blood. Thus, direct comparisons between the mouse and human system are difficult. DCs are a heterogeneous cell population that represents less than 1 % of the total cells in pheripheral blood [32].

DCs are derived from $CD34^+$ hematopoietic progenitor cells (HPCs). It has been proposed that the differentiation of DCs occur by two models. The first model postulated that a single committed DC lineage has functional plasticity depending on local environment signals, so called the “functional plasticity model”. The other

model proposed that distinct DC subsets are derived from multiple DC lineages in the early development , so called the “specialized lineage model” [36]. Depending on their developmental origin, cytokine activators, surface antigens, and functional capacity, DCs are subdivided into major distinct populations: conventional (cDC) and plasmacytoid DC (PDC) [32, 36] (Table III).

2.2.2.1.1.1 *Conventional DC*

Conventional DCs can be generated from the myeloid pathway and can be identified as lineage^{-}, CD11c^{+}, CD123^{dim}, and HLA-DR^{+} [37]. Under different conditions, CD11c^{+}HLA-DR^{+} precursor DCs, or CD14^{+}CD11c^{+} monocyte precursors isolated from the blood can be differentiated into two conventional DC subsets [32, 36, 38-41]: Langerhans cells (LCs), which are found in stratified epithelia such as the skin; and interstitial DCs, which are found in all other tissues [42]. LCs can be distinguished from interstitial DCs by the expression of Langerin and Birbeck granules [43]. Myeloid interstitial DCs (MDCs) are closely related to monocytes. For example, when cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), monocytes can be differentiated into MDCs. Furthermore, when monocytes or CD11c^{+} immature DCs are cultured with macrophage-colony-stimulating factor (M-CSF), they differentiate into macrophages. However, *in vivo,* the endometrium is influenced with the monocyte differentiation. Monocytes differentiated into DCs when they reverse transmigrate the endothelium in the ablumenal-to-lumenal direction whereas the remaining monocytes differentiated into macrophages [44].

MDCs are classical immunosurveillance cells. In normal steady state, immature MDCs reside in non-lymphoid organs and are characterized by effective Ag uptake. After endocytosing the antigen, MDCs become mature and acquire dendritic morphology, potent Ag presentation capacity (peptide-MHC complexed on the cell surface), and marked ability to induce Th cell responses [8, 45, 46].

2.2.2.1.1.2 *Plasmacytoid DC*

PDCs can be genereated from $CD11c^-$, $HLA\text{-}DR^+$ precursor DCs in the lymphoid pathway. PDCs can be identified as $lineage^-$, $CD11c^-$, $CD123^{bright}$, and $HLA\text{-}DR^+$ [37]. PDCs are the major type-1 IFN producing cells of human blood and are believed to play a role in antiviral responses [32, 45-48]. PDCs are found in the bone marrow, peripheral blood, and T cell areas of secondary lymphoid organs. They have lymphoid (plasma cell) morphology. Based on the nature of the antigen stimulus, PDCs can direct either Th1 or Th2 responses [49, 50].

Table III : Human DC subsets

Nomenclature	**Plasmacytoid DC**	**Interstitial DC**	**Langerhans cell (LC)**
Precursor in blood	CD11c-, CD123+	CD11c+, CD123- or CD14+, CD11c-	CD11c+, CD123- or CD14+, CD11c-
Phenotype	CD11c- CD123++ HLA-DR+ CD11b- Birbeck granule- Langerin-	CD11c+ CD123+ HLA-DR+ CD11b+ Birbeck granule- Langerin-	CD11c+ CD123+ HLA-DR+ CD11b+ Birbeck granule+ Langerin+
Localization	T-cell zones of lymphoid organs; DC precursors in blood	DC precursors in blood; Immature cells in tissue interstices	T-cell zones of lymphoid organs; DC precursors in blood; Immature cells in epithelia
Function IL-12(p70) secretion IFN-α secretion Phagocytosis	 +/- +++ -	 +++ - +++	 +++ - +++

Table content is based on (Pulendran B, et.al., 2004) [1].

2.2.2.1.2 *In vitro*

The DC population represents only a minute subpopulation in the peripheral blood mononuclear cells (PBMCs). In order to explore the usefulness of DCs in research studies, large numbers of these cells will be required, more than obtained by routine *ex vivo* purification methods [51].

Two difference precursor cells have been used to generate human DCs in culture. The earliest precursor cells is the $CD34^+$ HPCs isolated from bone marrow or umbilical-cord blood [52, 53]. The other one is peripheral blood monocytes [54-56], which is the most commonly precursor cell used for generating human DCs in culture. *In vitro* co-culture of these two precursor cells with GM-CSF and IL-4 can generated immature DCs within six days [54, 57]. These differentiated DCs are called hematopoietic progenitor DCs (HP-DCs) and monocyte-derived DCs (mo-DCs), respectively.

In vitro-generated DCs can be remained in the immature state. Upon stimulation by bacteria or bacterial products such as lipopolysaccharide (LPS), the immature DCs become mature by upregulation of MHC-II expression, costimulatory molecules and secretion of produce pro-inflammatory cytokines [58].

2.2.3.2 *Non-human primates*

Non-human primates (such as *rhesus macaque*) exhibit DC subsets with a similar phenotype and function as in humans [16-18]. Moreover, using similar protocols as in human, it is also possible to generate *in vitro* mo-DCs from non-human primates, specifically those for chimpanzee [59], cynomolgus monkeys (*Macaca fascicularis*) [60], and rhesus macaque (*Macaca mulatta*) [61].

2.2.3.3 *Mice*

2.2.3.3.1 *In vivo*

In mice, DCs are mainly isolated from lymphoid tissue. Several mouse DC subsets have been identified by using different markers as compared to human DCs. All mouse DCs express CD11c (the integrin-α_x chain) [62-65], whereas there is differential expression of the myeloid marker CD11b (the integrin α_M chain of Mac-1) and the lymphoid markers CD4 and CD8 [66]. CD8 on DCs (an αα-homodimer form) is different from CD8 on T cells (αβ-heterodimer, typical form). Although CD4 is also present on human DCs, CD8α is not. Another useful DC marker is CD205 (the multilectin domain molecule DEC205) [36].

At least six DC subtypes have been described in mice (Table IV). In the spleen three different DC subsets are found : (i) CD8α^- CD11b$^+$ CD4$^+$ DCs (so-called 'CD4$^+$ myeloid' DCs); (ii) CD8α^- CD11b$^+$ CD4$^-$ DCs (so-called 'CD8α^- CD4$^-$ DCs myeloid' DCs or double negative (DN)); (iii) CD8α^+ CD11b$^-$ DCs (so-called 'CD8α^+ lymphoid' DCs) [64]. These DCs reside in different compartments of the spleen, with CD11b$^+$ DCs mainly localized in the marginal zone and the CD8α^+ DCs mainly localized in the T-cell area of the white pulp [65, 67, 68]. In the lymph nodes, in addition to these three subsets, there are at least two extra DC subsets [62, 63] : (iv) CD8α^{dull} CD11b$^+$ CD205$^+$ CD4$^-$ Langerin$^+$ (so-called Langerhans cells-derived DCs (LCDCs)), and (v) CD8α^- CD11b$^+$ CD205^{lo} CD4- Langerin$^-$ (so-called 'dermal DCs'). The CD8α^+ subset is located in the T-cell areas of the spleen, lymph nodes, and Peyer's patches, and it can be induced to secrete abundant IL-12p70 and stimulate Th1 responses [69-72]. The CD8α^- DC subsets are located in the marginal zones of the spleen, the subcapsular sinuses of the lymph nodes, and subepithelial dome of the Peyer's patches; they produce minimal amouts of IL-12p70 and can induce Th2 responses [69-72]. The mouse LCDCs are located in epithelial tissue and migrate to the T-cell areas of the lymph nodes when they become mature [63, 73]. Moreover, mouse dermal DCs, they are resided in different tissue dermal layers [63].

In addition, recent studies described the discovery of precursor DC subset that have a plasmacytoid morphology and are able to secrete large amounts of IFN-α when stimulated with viruses or with CpG ODN [74-77]. This (vi) plasmacytoid precursor DC subset (or PDC precursor) has a phenotype of $CD11c^{dull}$ class II MHC^{dull} $B220(CD45)^{+}$ $CD8\alpha^{+}$ (subset) $CD4^{+}$ (subset) and has a the function similar to human PDCs. Mouse PDCs are scattered in the red pulp and the T-cell areas of the white pulp.

Table IV : Major DC subsets in murine secondary lymphoid organs

Nomenclature	**CD8α+ DCs**	**CD8α- DCs**	**Plasmacytoid DCs**	**Langerhans DCs**
Phenotype	CD11c+ Class II MHC+ CD8α+ CD205+ CD11b dull/- CD4- Birbeck granule- B220- Langerine-	CD11c+ Class II MHC+ CD8α- CD205- CD11b+ CD4+ or – [78] Birbeck granule- B220- Langerine-	CD11c dull Class II MHC dull CD8α+ or - CD205+ or - CD11b dull/- CD4- Birbeck granule- B220+ Langerine-	CD11c+ Class II MHC+ CD8α- CD205+ CD11b+ CD4+ Birbeck granule+ B220- Langerine+
Localization	T-cell zones of lymphoid organs; Thymic cortex	Marginal zones of spleen; Subcapsular sinus of lymph nodes; T-cell areas of lymph nodes; Sub-epithelial dome of Payer's patches	Marginal zones of spleen	Immature cells in epithelia; Mature Langerhans cells in T-cell zones of lymphoid organs
Function IFN-γ secretion IFN-α secretion IL-12p70 secretion	 ++++ + ++++	 - - +/-	 - ++++ -	 ? - ?

Table content is based on (Pulendran B, et.al., 2004) [1].

2.2.3.3.2 *In vitro*

As mentioned earlier, splenic mouse DCs are divided into “lymphoid” and “myeloid” based upon the expression of CD8α (CD8α^+ and CD8α^-, respectively) [66]. Recent studies revealed that DCs in mice can be expanded by fms-related tyrosine kinase 3 ligand (Flt3-L) and GM-CSF. Flt3-L can induce the expansion and differentiation of both lymphoid and myeloid DCs [69, 79, 80] whereas GM-CSF preferentially expands only the myeloid DC subset [70]. These effects can occured both *in vitro* and *in vivo.*

In vitro, a Flt3-L-dependent culture system can generate two bone-marrow (BM)-DC subsets [81] that have the different phenotypes and morphology. Whereas the “classical” $CD11c^+B220^-$DCs showed the classical stellate DC morphology as MDCs, $CD11c^+B220^+$ DCs appeared rounder and had a smooth surface with few dendrites and diffuse nuclei, an appearance similar to that of freshly isolated murine and human PDCs [74, 75]. Moreover, recent studies confirmed that the phenotype and function of BM-derived $CD11c^+B220^+$ DCs are similar to those of *in vivo* murine PDCs [74, 75, 82].

2.3 TOLL-LIKE RECEPTORS

The *Toll* gene of the fruit fly *Drosophila melanogaster* was the first member of the Toll family identified. It was discovered due to its essential function for the dorsoventral axis formation during embryogenesis [83]. As it is generally accepted that the *Drosophila* Toll in insect plays a role in immune responses to fungal infection [84], therefore, the identification of Toll-like receptors in mammals became subject to investigation.

2.3.1 TLR Structure

TLRs are type I transmembrane glycoproteins composed of two domains. The extracellular domain contains leucine-rich repeat (LRR) motifs that recognize conserved motifs of pathogens and a cytoplasmic domain, also known as the **Toll/IL-1R (TIR)** domain, that is similar to the corresponding domain of the interleukin-1 receptors (IL-1Rs) [85, 86]. This domain is crucial for signal transduction that leads to proinflammatory cytokine production.

2.3.2 TLRs and their ligands

TLRs, like other pattern recognition receptors (PRRs), recognize so-called pathogen-associated molecular patterns (PAMPs), which are conserved motifs that are unique to microorganisms [87]. To date, 10 TLRs (TLRs 1-10) have been identified in human as compared to 11 TLRs (TLRs 1-9 and 11-13) identified in mice [88]. From the homology database searches, some TLRs (TLR1-9) are similar between human and mouse, whereas others exhibit striking differences such as TLR10 and TLR11, that are not functional in mouse and humans, respectively [89].

A number of TLR ligands have been identified from various pathogens (Table V) [90-92]. TLR ligands can be classified into three categories based on their recognition products. TLRs 1, 2, 4 and 6 ligands are classified as lipid ligands and TLR5 and TLR11 ligands are protein ligands. There, these TLRs mainly recognize bacterial products. In contrast, the ligands for TLRs 3, 7, 8 and 9, which are localized in intracellular compartments [93-95], are classified as nucleic acid ligands. As TLRs have a distinct function in pathogen recognition, TLRs are a good target for rational adjuvant development.

Table V : Toll-like receptor ligands

Categories	Receptor	Ligand	Origin of ligand
Lipid Ligands	TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
		Soluble factors	*Neisseria meningitides*
	TLR2	Lipoproteins/lipopeptides	Various pathogens
		Peptidoglycan	Gram-positive bacteria
		Lipoteichoic acid	Gram-positive bacteria
		Lipoarabinomannan	Mycobacteria
		Glycoinositolphospholipids	*Trypanosoma cruzi*
		Zymosan	Fungi
		Atypical LPS	*Leptospira interrogans*
			Porphyromonas gingivalis
		Di- and triacyl lipopeptides	Synthetic compounds
		Heat-shock protein 70*	Host
	TLR4	Lipopolysaccharide	Gram-negative bacteria
		Taxol	Plants
		Heat-shock protein 60*	*Chlamydia pneumoniae*
		Heat-shock protein 70*	Host
	TLR6	Diacyl lipopeptides	*Mycoplasma*
		Lipoteichoic acid	Gram-positive bacteria
Protein Ligands	TLR5	Flagellin	Bacteria
		Discontinuous 13-amino-acid peptide	Synthetic compounds
	TLR11	Not done	Uropathogenic bacteria
Nucleic acid Ligands	TLR3	Double-stranded RNA	Viruses
		Poly I:C	Synthetic compounds
	TLR7	Imidazoquinoline	Synthetic compounds
		Loxoribine	Synthetic compounds
		Bropirimine	Synthetic compounds
		Single-stranded RNA	Viruses
	TLR8	Imidazoquinoline	Synthetic compounds
		Single-stranded RNA	Viruses
	TLR9	CpG-containing DNA	Bacteria and viruses
		CpG oligodeoxynucleotides	Synthetic compounds
Other	TLR10	Not done	Not done

2.3.2.1 TLR1, TLR2 and TLR6

TLR2 recognizes the bacterial lipoproteins by forming both homodimer and heterodimers with TLR1 and TLR6 [5]. Homodimers of TLR2 recognize various gram-positive bacterial motifs such as peptidoglycan, lipotechoic acid (LTA); mycobacterium motifs such as Lipoarabinomannan; parasitic motifs such as glycoinositolphospholipids in *Trypanosoma cruzi*; fungal motif such as zymosan; and atypical LPS found in gram negative bacterial species such as *Leptospira interrogans* and *Porphyromonas gingivalis* [5, 96, 97]. Moreover, TLR2 has also been demonstrated to recognize self antigens such as Heat Shock Protein 70 (HSP70) [5, 96, 97].

Heterodimerization of TLR2 and TLR1 resulted in the recognition of tri-acyl lipoproteins that are primarily found in mycobacterium as well as soluble factors from *Neisseria meningitides* [5, 96, 97]. In addition, heterodimerization of TLR2 and TLR6 resulted in the recognition of di-acyl lipoproteins such as MALP-2 found in mycoplasma species [5, 96, 97]. Studies in mice indicated that in the absence of TLR2, mice exhibits greater susceptibility to gram-positive bacteria and spirochetes such as *Borrelia burgdorferi* [5, 96, 97].

2.3.2.2 TLR3

TLR3 is involved in recognition of dsRNA motifs, which are the viral replicative forms [98]. The most commonly used TLR ligand is synthetic double-stranded polyriboinosinic:polyribocytidylic acid (Poly I:C), which has a similar activity to that of dsRNA [99]. TLR3 has been demonstrated to be involved in the antiviral response to Mouse Cytomegalovirus (MCMV) and human Influenza A virus [19, 100-102]. In mice, TLR3 mediates entry of West Nile Virus into the brain leading to lethal encephalitis [103]. In parallel, human TLR3 mediates responses to respiratory syncytial virus (RSV) [100, 104, 105]. The ligation of TLR3 has also been demonstrated to be important as a therapeutic agent. Use of Poly I:C as an

adjuvant increases vaccine efficacy for viruses such as HSV-2, Hepatitis B, and Influenza A viruses [19, 106].

2.3.2.3 TLR4

TLR4 forms a homodimer in recognition of bacterial LPS [5, 96, 97]. LPS is major component of the outer membrane of gram-negative bacteria [107] that mainly consists of lipid A. A glycosylphosphatidylinositol (GPI)-anchoring protein, CD14, was identified to facilitate LPS action by binding and retaining LPS on the cell surface. This suggests that another membrane proteins may be essential for LPS signaling. Although low dose of LPS can induce the immunostimulatory responses, high dose of LPS can cause a clinically life-threatening condition called endotoxin shock. In addition, TLR4 is involved in the recognition of taxol, a diterpene purified from the bark of the western yew (*Taxus brevifolia*) [108, 109]. Furthermore, TLR4 has been shown to be involved in the recognition of endogenous ligands, such as HSP60 and HSP70, the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen.

2.3.2.4 TLR5

TLR5 has been demonstrated to recognize bacterial flagellin, a monomeric constituent of bacterial flagella [110, 111]. The flagella from several bacteria species can be recognized by TLR5 such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Escherichia coli* [112, 113]. In human gut, TLR5 expression is on the basolateral, but not the apical side of intestinal epithelial cells [114]. In addition, flagellin activates lung epithelial cells to induce inflammatory cytokine production [115]. These findings illustrate the essential role of TLR5 in microbial recognition at the mucosal surface. Moreover, a TLR5 polymorphism, in which a stop codon occurs in the ligand-binding domain of TLR5, has been shown to be associated with susceptibility to *Legionella pneumophila* infection [115].

2.3.2.5 TLR7 and TLR8

Because TLR7 and TLR8 have strong homology of their structures, they can recognize the same ligands in some cases. Recent studies revealed that synthetic antiviral compounds such as imidazoquinoline-like molecules including imiquimod (R-837), resiquimod (R-848), S-27609, and guanosine analogues such as loxoribine can be recognized by both TLRs [93, 116-118]. In mice, TLR7 is activated by all of these compounds, but TLR8 is not [93, 119]. However, in human, TLR8 is activated by resiquimod [93, 120]. Because these synthetic antiviral compounds are structurally related to guanosine nucleoside, it was not surprising that the natural ligands for TLR7 and TLR8 were identified to be viral ssRNA species such as human immunodeficiency virus, vesicular stomatitis virus and influenza virus [90-92].

2.3.2.6 TLR9

TLR9 is involved in both antiviral and antibacterial responses. TLR9 recognizes unmethylated CpG motifs that are present in microbes, but rare in mammalian species [78]. In humans, there are three types of CpG motif (GTCGTT) that initiate TLR9-mediate responses [96, 121]. CpG-A motifs (also known as D-type) induce high amounts of IFN-α, especially from PDCs, but induce low level of IL-12. CpG-B motifs (also known as K-type) are potent inducers of inflammatory cytokines such as IL-12 and TNF-α, especially from B cells. CpG-C motifs mediate the effects seen by both CpG-A and CpG-B [121-125]. However, the recognition of CpG motif is species-specific. Mouse and humans TLR9 require slightly different sequence CpG motifs [126, 127]. Recently, CpG DNA was shown to be useful as an adjuvant. CpG DNA-conjugated proteins have been shown to promote both the antigen presenting activity and the maturation of DCs, thereby enhance the antigen-specific Th1 responses [128].

2.3.3 TLR distribution

TLR are expressed differentially among the immune cells of both innate and adaptive immunity. On innate immune cells, TLRs are expressed on neutrophils, macrophages, DCs, dermal endothelial cells and mucosal epithelial cells. In addition, TLRs can be found on the cells involved in adaptive immunity including B an T cells [129]. Furthermore, TLRs are also found on mucosal epithelium and structural cells of tissues such as fibroblasts [130].

2.3.4 TLR Function

TLRs function to initiate the immune system to fight the invading microorganisms. Engagement of TLRs with their ligands leads to the production of various pro-inflammatory cytokines, chemokines, and effector molecules, depending on the cell type that is activated [131-133].

2.3.4 TLR Signaling Pathway

Stimulation of TLRs by microbial components triggers signal transduction cascades leading to expression of immune response genes that function in host defence, including inflammatory cytokines, chemokines, MHC and co-stimulatory molecules. All TLRs contain a TIR signaling domain that interacts with the TLR adaptor molecules. For simplicity only the adaptor proteins, MyD88 (Myeloid differentiation factor 88) and TRIF (TIR-domain-containing adaptor protein inducing IFN-β) will be described here.

Two intracellular signaling pathways are activated by TLR-stimulation (see Figure 2.1) [134, 135]. A common signaling pathway through the adaptor protein MyD88 (MyD88-dependent pathway), which is used by all TLRs except TLR3, leads to activation of NF-κB and MAP kinases that ultimately results in expression of pro-inflammatory cytokines like TNF-α, IL-6 and IL-12. TLR3 (and also TLR4)

signals through the adaptor-protein TRIF (MyD88-independent/TRIF-dependent pathway), leading to activation of interferon-regulatory factor-3 (IRF-3), IRF-7 and NF-κB, which together activate the IFN-α/β promoter. It should be noted that TLR7 and 9 can also induce IFN-α production through activation of IRF-7 [136].

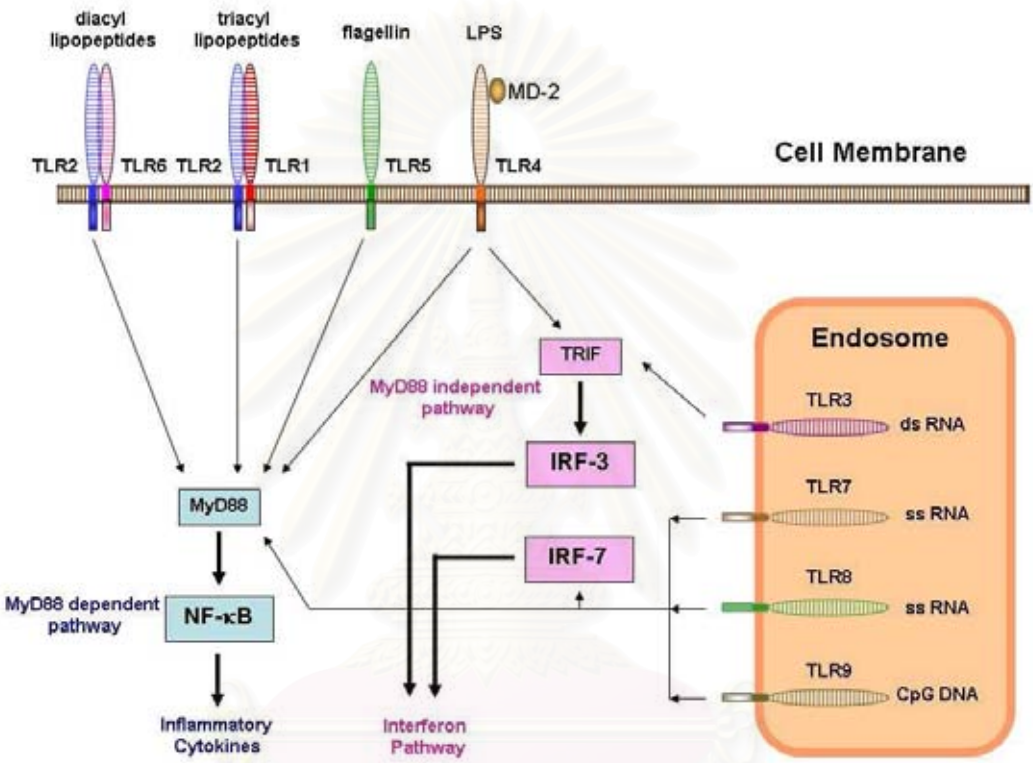


Figure 2.1 : Summary of TLR ligands and their signaling pathways.

TLRs are transmembrance proteins that are localized on the cell membrane or in endosomes. Triggering of TLRs leads to a signaling cascade that finally induces the inflammatory cytokines through a MyD88 pathway or interferons through a MyD88 independent pathway.

2.3.6 The distribution of TLR expression on DCs and other APCs

The examination of DC subsets isolated from humans and mice has been indicated that TLRs have distinct expression patterns (Table VI). In human, PDCs express TLR 7 and TLR9, whereas MDCs express TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR8 [10, 12, 13]. There are some studies that reported that TLR7 was expressed in both PDC and MDCs [11, 137], whereas others found that TLR7 was expressed in PDCs only [12, 13]. Human blood monocytes express TLRs 1, 2, 4, 5 and 8, but progressively lose these receptors as they differentiate into immature mo-DCs in the presence of GM-CSF and IL-4 and instead acquired the expression of TLR3 [138]. Notably, human MDCs and *in vitro*-differentiated immature mo-DCs express TLR3 in their intracellular compartments [94], unlike human fibroblasts, which express TLR3 on the cell surface. Human B cells and PDCs express TLR9, which is not found in monocytes, mo-DCs and MDCs [11-13].

The reason for the difference of TLR expression between human and mouse DC subsets remains unclear; a possible explanation could be the different sources of DCs. Human DC subsets are purified from peripheral blood whereas mice DCs are mainly isolated from secondary lymphoid organs. In mice, all splenic DC subsets express TLRs 1, 2, 4, 6, 8, and 9 [15]. However, mouse PDCs do not express TLR3. In comparison to human, although mouse spleen PDCs express TLR7 and TLR9, they also express the other TLRs [15]. Moreover, mouse $CD8\alpha^+$ DCs do not express TLR5 and TLR7 and fail to respond to TLR7 agonists [15, 139]. The study of TLRs expression in *in vitro* mouse BM-DCs culture revealed that BM-derived $CD11c^+B220^-$ and $CD11c^+B220^+$ DCs exhibit substantial expression levels of TLRs 2, 3 , 4, 7 and 9 [82]. The “Classical” $CD11c^+B220^-$ BM-derived DCs preferentially expressed TLRs 2, 3 and 4, whereas the expression of TLRs7 and 9 was higher in BM-derived and in freshly isolated splenic $CD11c^+B220^+$ PDCs [82]. One major difference should be noted. TLR9 is expressed by all mouse DC subsets, either from spleen or from *in vitro* BM-DCs, as well as macrophages and B cells [15]. In

human, TLR9 is expressed in PDCs and B cells only. These differences affect the validity of mouse models using TLR9 ligands. The direct accessment of mouse data to human should be taken into consideration.

2.3.7 TLR-dependent DC-mediated control of adaptive immune response

Recognition of microbial components by TLRs, which are highly expressed on DCs, triggers activation of not only innate immunity but also adaptive immunity. Recognition of PAMPs through TLRs leads to the induction of two signals that are important for T cells activation and induction of adaptive immunity. Signal 1 is the peptide-MHC complex and signal 2 is the co-stimulatory signal. Therefore, the activation status of the antigen presentation plays a role for making T cells become either immunogenic or tolerant (anergic). The co-stimulatory signal that is induced by TLRs is one of the mechanisms for discrimination of self from non-self [4, 8].

Table VI : Summary of TLR expression in Human and Mouse APCs

Species	Type of cells	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	Reference
Human	**PDCs**	+[a]	-[b]	-	-	-	+	+	-	+	[10-13, 94, 137]
	MDCs	+	+	+	+	+	+	+/-	+	-	[11-13, 137]
	mo-DCs (*In vitro*)	+	+	+	+	+/-	+	-	+	-	[13, 94, 138, 140]
	Monocytes	+	+	-	+	+	+	+/-	+	-	[10-13, 137, 138, 140]
	B cells	+	+	-	+	-	+	+	+/-	+	[10, 141, 142]
Mouse	**PDCs**	+	+	-	+/-	+	+	+	+	+	[15, 143]
	$CD4^+$	+	+	-	+/-	+	+	+	+	+	[15, 143]
	$CD8\alpha^+$	+	+	+	+/-	-	+	-	+	+	[15, 143]
	DN	+	+	+	+/-	+	+	+	+	+	[15, 143]
	$CD11c^+B220^-$ (*In vitro*-BM-DCs)		+	+	+			+	+		[82]
	$CD11c^+B220^+$ (*In vitro*-BM-DCs)		+	+	+			+	+		[82]

a indicate the absence of mRNA expression of each TLRs
b indicate the presence of mRNA expression of each TLRs

2.4 ADJUVANTS IN VACCINE RESEARCH

2.4.1 Evolution of vaccines

Live attenuated vaccine, the weakened strains of a pathogen, is the earliest vaccine development. Although it can stimulate an effective immune response, several risks should be noted such as it can cause disease in the people who have a weak immune response. Therefore, the trend of vaccine development has been moving towards the “subunit vaccines”, which can be produced as highly purified antigens. Since subunit vaccines cannot replicate in the host, there is no risk of disease. However, subunit vaccines do not induce strong immune responses. As a result of this, potent adjuvants are required to make such vaccines more immunogenic.

2.4.2 Adjuvants

Adjuvants are defined as heterogenous components that enhance or modulate the vaccine immunogenicity [144]. The role of innate immunity in stimulating adaptive immune responses is the basis of the action of adjuvants. Therefore, adjuvants are an essential part of vaccines.

Over the past decades, the gold-standard adjuvant composed of a mixture of mineral oil and heat killed mycobacteria is known as Complete Freunds adjuvant (CFA). CFA exhibits highly effective vaccine responses in animals, but it is not useful in human vaccination due to it toxicity [145]. The same toxicity effects were also observed using incomplete Freunds adjuvant (IFA), which is the oil component alone (ref). To date, there are only two licensed adjuvants for human use including aluminum salt (alum) and squalane oil/water emulsion (MF59). Alum is an aluminum-based mineral salt, which was first discovered in 1926. Alum is still the most widely used adjuvant in human. Because alum consists of a gel-like insoluble form, it adsorbes with the antigen and forms a ‘depot’ effect at the vaccination site.

Unfortunatly, alum is a poor adjuvant because it induces only strong Th2 responses [146].

As mentioned above, a good vaccine adjuvant should induce both innate and adaptive immunity. It is well known that innate immunity can easily be triggered by TLR stimulation leading to strong adaptive immunity. A recent study of the live attenuated yellow fever vaccine 17D, one of the most effective vaccines, revealed that multiple TLRs are involved in the generation of the adaptive immune response [147]. Nowadays, almost all of the TLR ligands have been evaluated as adjuvants and shown to enhance the vaccine efficiency in animal studies. Moreover, TLR4 and TLR9 ligands were shown to enhance the vaccine immunogenicity in human clinical trials [148-150].

Although LPS, the TLR4 ligand, is a potent vaccine adjuvant, it caused the toxic effect in humans [151, 152]. The chemical derivatives of the lipid A were modified for reducing its toxicity. The monophosphoryl lipid A (MPL) has been used widely in combination with the vaccine in clinical trials. Recently, one of the modified MPL derivatives, “RC-529 or Ribi Adjuvant”, has been used in a hepatitis B vaccine trial. The similar protective antibody level was obtained by using only two doses of the adjuvants mixed with vaccine comparing with three doses of vaccine alone [148]. This formulation has been licensed in Europe (Fendrix).

As TLR9 agonist, CpG 7909, which is one of the CpG-ODNs, has been tested and shown to enhance the protective effects of vaccines in human clinical trials [149, 150]. CpG 7909 mixed with hepatitis B vaccine induced faster and higher specific antibody responses compared with the vaccine alone [153]. Moreover, combining CPG 7909 with IFA in a tumor vaccine showed that higher specific CTL responses can be induced by this adjuvant [154].

2.5 WHICH PRECLINICAL MODEL ?

For evaluation of adjuvant development, the predictability of animal models is questionable in some circumstances. The most widely used preclinical model are mice, but although they often generate helpful information, there are some significant differences between the immune systems of mice and humans [155]. As stated above, TLR9 is expressed on different DC subsets in mice and humans and optimal CpG sequences are also species-specific. Moreover, the responsiveness of TLR8 is also weak in mice [156].

Based on their close relationship to humans, non-human primates such as *rhesus macaques* have proven to be valuable as animal models for testing vaccines and immunization strategies [157, 158]. Also, several reports showed the presence of MDCs and PDCs in circulation in macaques [16-18]. Moreover, several groups have used TLR ligands as adjuvants and evaluated their effects in macaque models, including immunization of the antigen together with TLR3 [19], TLR7/8 [20], or TLR9 ligands [21]. However, so far, it is not known if a similar set of TLRs is present on APCs from human and macaque. Therefore, the better understanding of TLR expression and also their function in preclinical animal models is important in the evaluation of TLR adjuvant development.

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

For comparative study, targeted antigen presenting cells were derived from three species as described:

3.1.1 **Non-human primate** : Rhesus Macaques (*Macaca mulatta*) were housed at the Armed Forces Research Institute of Medical Sciences (Bangkok, Thailand).

3.1.2 **Human** : Peripheral venous blood from healthy donors were derived from the Armed Forces Research Institute of Medical Sciences (AFRIMS) and buffy coats were derived from Thai Red Cross Blood Bank (Bangkok, Thailand).

3.1.3 **Mice** : BALB/c mice, 8-12 wk of age, were purchased from National Laboratory Animal Centre, Mahidol University (Bangkok, Thailand).

3.2 METHODS

3.2.1 PURIFICATION AND GENERATION OF DC SUBSETS AND OTHER APCS FROM : MACAQUE, HUMAN, AND MOUSE

3.2.1.1 Purification of macaque and human dendritic cell subsets

Peripheral venous blood was obtained from healthy human donors and adult rhesus macaques (*Macaca Mulatta*) maintained in accordance with guidelines of the Institutional Animal Care and Use Committee. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). T cells were removed by rosetting with neuraminidase-treated sheep red blood cells [17], in case of cell isolation by flow cytometry.

3.2.1.1.1 Isolation of macaque DC subsets

Flow cytometric cell sorting (FACS) was used to isolate DCs for TLR expression assay whereas immunomagnetic cell sorting (MACS) was used to isolate DCs for TLR functional assay. The purity of all sorted DC populations was >95% by FACS and >80% by MACS as determined by FACSCalibur (BD Biosciences).

For mRNA expression study, T cell-depleted populations were stained with Ab against CD20 (FITC) (BD Biosciences (San Jose, CA)) and CD1c (Anti-BDCA-1) (PE) (Miltenyi Biotec, Germany) for **MDCs** isolation and stained with mAbs against HLA-DR (FITC) (BD Biosciences (San Jose, CA)) and CD123 (PE) (BD PharMingen (San Diego, CA)) for **PDCs** isolation. Then, $CD20^{-}$ and $CD1c^{bright}$ cells (MDCs) and $CD123^{bright}$ $HLA\text{-}DR^{+}$ cells (PDCs) were sorted with a FACSVantage (BD Biosciences, Mountain View, CA). After isolation, cells were spined down and keep at -80ºC immediately for preventing cell activation.

For functional study, highly pure **CD1c$^+$ (or BDCA-1) (MDCs)** without contaminating CD1c$^+$ B cells were obtained by immunomagnetic depletion of CD20$^+$ B cells using CD20 mAb-conjugated microbeads (Miltenyi Biotec, Germany) followed by immunomagnetic enrichment of CD1c$^+$ MDCs. For macaque **PDCs**, CD123-expressing cells were isolated from PBMCs by indirect magnetic labeling with PE-conjugated mAb and anti-PE mAb-conjugated microbeads (Miltenyi Biotec) and enrichment of labeled cells by MACS. The purity was analyzed by counterstaining with HLA-DR.

3.2.1.1.2 Isolation of human DC subsets

Human **MDCs** were purified by staining T cell-depleted populations with Lin FITC (lineage markers: CD3, CD4, CD16, CD56 and CD20), and CD11c-PE (BD Biosciences (San Jose, CA)); Lin$^-$ and CD11c^{bright} cells were sorted. The sorted cells were analyzed and were found to express HLA-DR, but minimally expressed CD123 and had typical myeloid morphology. For **PDC** purification, human T cell-depleted populations were stained with mAbs against HLA-DR (FITC) and CD123 (PE), and CD123^{bright} HLA-DR$^+$ cells were sorted. The sorted cells had a typical plasma cell-like morphology and did not express either CD11c or lineage markers.

3.2.1.2 Generation of macaque and human monocyte-derived dendritic cells (mo-DCs)

CD14$^+$ monocytes were isolated by positive selection from PBMCs using non-human and human primate CD14 magnetic cell sorting (MACS) system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

- In brief, 10^7 PBMCs were incubated with 80 μL MACS buffer (For rhesus macaque: Endotoxin free 1XPBS and 1% fetal calf serum (FCS); for human: 1XPBS, 1% FCS and 2mM EDTA) and 20 μl of CD14 MicroBeads and kept on ice for 15 min.

- Wash cell by adding 1 mL of MACS buffer and centrifuge at 300xg for 10 minutes.

- After washing off the excess beads, purified CD14+ were passed through an MS MACS separation column, according to manufacturer's instructions.

3.2.1.2.1 Generation of macaque mo-DCs

Macaque monocytes ($1.25x10^6$ cells/mL) were culture in complete medium (CM) consisting of RPMI 1640 supplemented with non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/mL penicillin, 100 μg/mL streptomycin (Gibco Laboratories, Grand Island, NY) and 10 μg/mL β-mercaptoethanol, supplemented with 1% FCS for 6 days in the presence of 100 ng/mL of recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL of rh interleukin (IL)-4 (both from R&D System, Minneapolis, MN). Every second day, half the volume of medium was replaced with fresh medium containing the same amount of cytokines.

3.2.1.2.2 Generation of human mo-DCs

Human monocytes were cultured at $1x10^6$/mL in CM supplemented with 10% FCS in the presence of 100 ng/mL rh GM-CSF and 100 ng/mL rh IL-4. All cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2. Every second day, half the volume of medium was replaced with fresh medium containing the same amount of cytokines.

3.2.1.3 Purification of macaque and human monocytes and B cells

$CD14^+$ monocytes and $CD20^+$ B cells were isolated by positive selection from PBMCs using non-human and human primate CD14 and CD20 magnetic cell sorting system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

- In brief, 10^7 PBMCs were incubated with 80 μL MACS buffer (For rhesus macaque: Endotoxin free 1XPBS and 1%FCS; for human: 1XPBS, 1% FCS and 2mM EDTA) and 20 μl of CD14 or CD20 MicroBeads and kept on ice for 15 min.

- Wash cell by adding 1 mL of MACS buffer and centrifuge at 300xg for 10 minutes.

- After washing off the excess beads, purified $CD14^+$ or $CD20^+$ were passed through an MS MACS separation column, according to manufacturer's instructions.

3.2.1.4 Generation and purification of mouse bone marrow-derived dendritic cells (BM-DCs)

Mouse bone marrow (BM) cells were isolated from femurs and passed through nylon mesh. BM cells were the cultured in CM supplemented with 5% FCS and 100 ng/ml recombinant mouse Flt3-L (R&D System, Minneapolis, MN) at 10^6 cells/mL and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 7 days. On day 4, half the volume of medium was replaced with fresh medium containing the same amount of cytokine.

On day 7, cells were harvested, stained with PE-labeled anti-CD11c and FITC-labeled anti-B220 (BD Biosciences (San Jose, CA)) and sorted by FACSVantage Cell sorter (BD Biosciences, Mountain View, CA) into $CD11c^+$ $B220^-$ (MDCs) and $CD11c^+$ $B220^+$ populations (PDCs) (purity > 95%).

3.2.2 COMPARATIVE TLR EXPRESSION ON DC SUBSETS AND OTHER APCS FROM : MACAQUE, HUMAN, AND MOUSE

3.2.2.1 RNA Extraction

Total RNA was isolated from various DC subsets and other APCs ($3x10^5$-$10x10^5$ cells) using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The procedure was based on a spin column, with the ability to bind RNA and exclude DNA or protein contaminants.

To avoid contamination with DNA, extracted RNA was treated with RQ1 RNase-free DNase (Promega Corporation) for 30 min at 37°C. All RNA preparations were standardized by RT-PCR for β-actin and were free from DNA contamination as judged by a lack of signal from non-reverse transcribed RNA with all primer sets.

3.2.2.2 cDNA Synthesis

cDNA was synthesized using oligo $(dT)_{18}$ primers and the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada). 1 µg of RNA was incubated with 1mM dNTP mix, 0.5 µg oligo $(dT)_{18}$ primers, 20 U RiboLock™ Ribonuclease inhibitor and 200 U RevertAid™ M-MuLV reverse transcriptase all diluted in 1x reaction buffer, at 42°C for 1 min. The reaction is inactivated by boiling at 70°C for 10 min. The cDNA produced was stored at -20°C.

3.2.2.3 Semi-Quantitative RT-PCR optimization

For semi-quantitative RT-PCR, one set of TLR 3, 4, 7, 8 and 9 and intracellular positive control, beta-actin primers was designed for all three species. All sequences were retrieved from NCBI database and primers were designed using an on-line primer-design program named Primer3 Output

(http://wwwgenome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Table VIII (Appendix I) shows the mRNA accession code from the NCBI database, the primer sequences with their expected product lengths, optimal annealing temperature, and optimal thermal cycles. All primers were synthesized by Proligo, Sigma.

Appendix I, Part I showed the sequence alignment of all three species for each target genes by ClustalW program (BioEdit, Ibis Biosciences, Carland, CA). Primers were designed based on conserved regions of TLRs. Afterwards, all the primers were analysed for their specificity by NCBI BLAST.

Appendix I, Part II shows the PCR optimization. Cells from each species were used for PCR optimization. For mouse, spleen cells were used for optimizing all the primers. For human and macaque, PBMCs were used for optimizing the TLR4, 7, 8 and β-actin primers whereas mo-DCs and B cells were used for optimizing TLR3 and TLR9, respectively. Firstly, each primer set was optimized for its *annealing temperature (Ta)*. The starting Ta was chosen based on the calculation of length and composition of the primers. Then, the Ta was optimized by varying the temperature ± 5 ºC from the calculated Ta. An optimal Ta was chosen from the temperature that gives specific amplification and has high yields of a unique product. Secondly, the *cycle number* was optimized, by obtaining PCR samples every five cycles between 25X and 45X thermal cycles. The cycle number was chosen within the exponential rate of product accumulation and before it reached plateau. Finally, the sensitivity of each primer set was done by varying the amount of input cells (20,000, 10,000 and 5,000 cells) per reaction.

All cDNA from APC subsets were performed under the optimized PCR condition in Appendix I, Table IX by using a GeneAmp PCR System 9700 (PerkinElmer/Applied Biosystems). Corrected size and yield of PCR products were analyzed on a 1.5% agarose gel.

3.2.3 TLR FUNCTIONAL STUDIES IN MACAQUE APCS AND HUMAN MONOCYTE-DERIVED DCS

3.2.3.1 *In Vitro* Stimulation

For assessment of TLR function, the various macaque DC cell populations, other APCs and human mo-DCs were seeded into 96-well flat bottom plates at $5x10^5$-$1x10^6$ cells/mL in CM supplemented with 10% FCS for macaque PDCs, MDCs, monocytes, B cells and human mo-DCs and 1% FCS for macaque mo-DCs and then stimulated in the presence or absence of the indicated TLR ligands below for 24 h. Optimal concentrations of TLR ligands were obtained by titration of different concentrations in initial experiments and subsequently used at a concentration that caused significant DC phenotypic differentiation without increased cell death.

- TLR3 ligand : 100 μg/mL dsRNA complex polyinosine-polycytidylic acid (Poly(I:C)) (Invivo-Gen (San Diego, USA)).

- TLR4 ligand : 5 μg/mL Ultra Pure *Escherichia coli* K12 LPS (Invivo-Gen (San Diego, USA)).

- TLR 7 ligand : 3 μg/mL Imiquimod (R837) (Invivo-Gen (San Diego, USA)).

- TLR8 ligand : 5 μg/mL single-stranded polyU oligonucleotide complexed with LyoVec^{TM} (ssPolyU/LyoVec) (Invivo-Gen (San Diego, USA)).

- TLR9 ligand : 5 μg/mL CpG-oligodeoxynucleotide (ODN) 2006 (TCGTCGTTTTGTCGTTTTGTCGTT) that was obtained from Coley Pharmaceutical Group (Wellesley, MA).

The J558L transfected with CD40L fibroblast cell line was additionally used to stimulate macaque mo-DCs [159].

3.2.3.2 Phenotypic Analysis

To evaluate surface expression levels of maturation markers and costimulatory molecules, cultured cells were harvested and washed in PBS supplemented with 0.5% BSA. Then, cells were stained with combination of FITC-labeled CD40, PE-labeled CD83 and APC-labeled CD86 and analyzed by a FACSCalibur flow cytometry (BD Biosciences). Nonspecific Ab binding was also examined by staining the cells with fluorochrome-labeled isotype-matched normal mouse Igs.

3.2.3.3 Quantitation of Cytokine Production

To evaluate cytokine production, the culture supernatants were collected and analyzed by the macaque-reactive IL-12(p70), TNF-α ELISA (Biosource International, Camarillo, CA), macaque cross-reactive human IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ), human-reactive IL-12p70 and the human-reactive TNF-α (R&D Systems, Minneapolis, MN). All assays were performed according to the manufacturer's instructions.

3.3 STATISTICAL ANALYSIS

Data are depicted as mean ± SD. A two-tailed student's *t* test for paired samples was used for comparison of unstimulated APCs to APCs that were stimulated with different TLR ligands (TLR3, 4, 7, 8 and 9) using Prism version 3.0; GraphPad Software, Inc., San Diego, CA). A "p" value of 0.05 or less was considered to be signicant (*).

CHAPTER IV

RESULTS

4.1 PURIFICATION AND GENERATION OF DC SUBSETS FROM MACAQUE, HUMAN AND MOUSE

4.1.1 Isolation and phenotypic characterization of macaque dendritic cell subsets

Dendritic cells represent a heterogeneous population consisted of several subsets [8]. In human blood, PDC and MDC subsets comprise approximately 1%-1.5% of total PBMCs, making them difficult to isolate and study in large number. However, specific combinations of antibodies enable isolation of these subsets using cell sorting.

We set out to isolate macaque blood DC subsets using anti-human mAbs known to cross-react with this species. Two techniques of cell sorting were used to isolate DC subsets depending on the purpose of studies. Because of the purity of sorted DC populations by flow cytometric cell sorting (FACS) was more than 95%, therefore the FACS technique was used to isolate DCs for mRNA expression study. However, the yield of isolated cells relative to starting PBMCs was low and the process of isolation can cause the mechanical stress to the cells. Therefore, immunomagnetic cell sorting (MACS) was used to isolate DCs for TLR functional study instead.

Using the same two-color FACS analyses used to identify human PDCs, macaque PDCs were isolated by FACS sorting of HLA-DR^{+} CD123^{bright} cells and thus phenotypically resemble human PDCs (Figure 4.1). Interstingly, the mean fluorescence intensity (MFI) of expression of HLA-DR in macaque PDCs showed more variation than in human PDCs. Moreover, macaque PDCs showed only one

homogenous group of CD123 staining. However, for TLR functional study, antibodies to human PDC-specific surface markers BDCA-2 and -4 did not cross-react with macaque PDCs [160]. Therefore, we initiated anti-PE microbeads to sort CD123-PE positive cells from PBMCs and it showed more than 80% purity (Figure 4.2). Freshly sorted PDCs showed a uniform appearance with eccentric reniform nuclei, prominent Golgi region, and numerous short, veil-like projections.

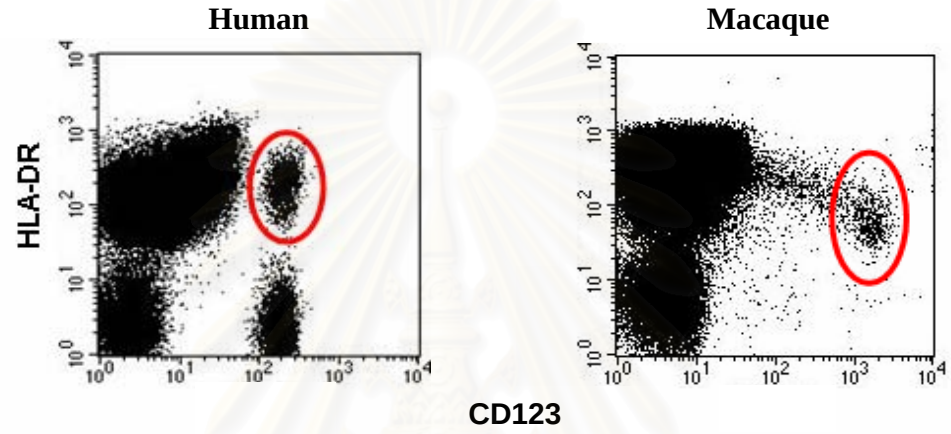


Figure 4.1 : Two-color flow cytometry gating identifies macaque PDCs (right panel) that phenotypically resemble human PDCs (left panel). PBMCs were first selected based on forward and side scatter and then further defined as $CD123^+$ and $HLA\text{-}DR^+$ cells. The data are representative of typical flow cytometric analysis.

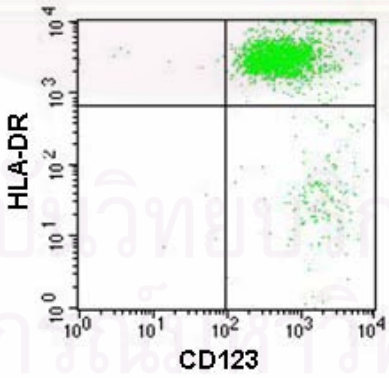


Figure 4.2 : Fluorescence-activated cell sorting analysis shows the purity of $CD123^+$ and $HLA\text{-}DR^+$ macaque PDCs by magnetic cell sorting (MACS) system. Anti-PE microbeads were used to sort CD123-PE positive cells from PBMCs. The purity was more than 80%. The data shown is one representative of three independent experiments.

For MDCs, human MDCs can be purified by two-color analysis as Lin-$CD11c^+$ cells. However, these antibody specific markers did not cross-react with macaque MDCs as shown in Figure 4.3. Therefore, BDCA-1 (a myeloid marker that identifies CD1c in humans) was replaced as an alternative marker for identify macaque MDCs. Macaque MDC were isolated as $CD1c^+$ $CD20^-$ cells using FACS sorting (Figure 4.4) or magnetic beads sorting. Freshly isolated MDCs showed reniform or multilobulated nuclei, with few dendrites and veil-like cytoplasmic projections.

In whole blood, we identified approximately 1231 ± 555 PDCs/10^6 PBMCs (0.1%, n =18 donors) and 6894 ± 3659 MDCs/10^6 PBMCs (0.7%, n = 17 donors), considerably less compared to human blood PDCs and MDCs [32].

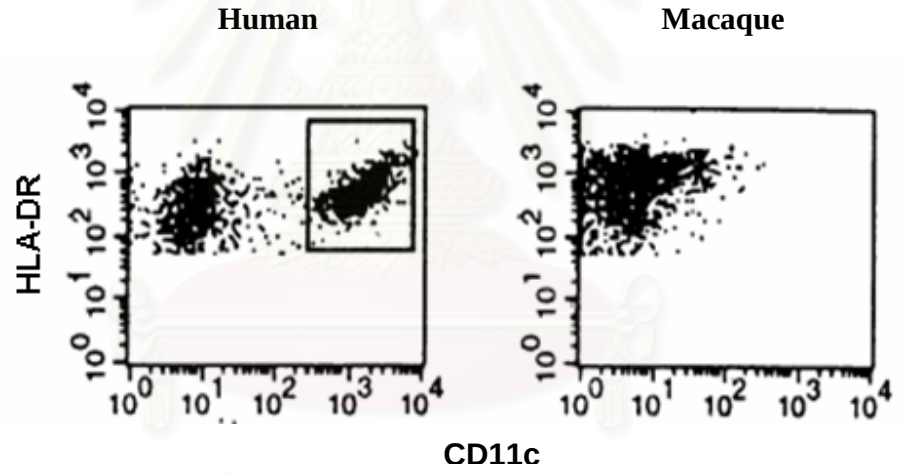


Figure 4.3 : Human MDCs were clearly identified by anti-CD11c whereas macaque MDCs were not. In fresh human and macaque PBMCs, anti-CD11c (clone 3.9 (Biosource, CA), react with macaque cells) did not stain intensively enough to allow macaque MDCs separation. Data are representative of six monkeys.

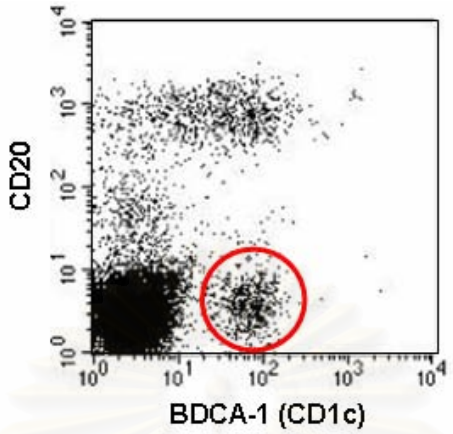


Figure 4.4 : Identification of macaque MDCs using the CD1c (BDCA-1).
Using FACS sorting, Macaque PBMCs were first selected based on forward and side scatter and then further defined as CD1c (BDCA-1)$^{+}$ and CD20^{-} cells. The data are representative of typical flow cytometric analysis.

4.1.2 Generation and characterization of macaque monocyte-derived DCs

Large numbers of DCs can be generated *in vitro* from macaque CD14^{+} peripheral blood monocytes by culture with GM-CSF and IL-4 [54] and were similar to that of human CD14-derived DCs. However, as compared to human mo-DCs, a modified method was established for the generation of rhesus macaque mo-DCs, using similar amounts of GM-CSF (100 ng/ml), but 10 times lower amounts of IL-4 and only 1% FCS. This method gave rise to mo-DCs with a typical DC morphology. We observed that macaque cytokine-generated DCs are easily activated by trace amounts of LPS, requiring endotoxin-free isolation and culture conditions.

4.1.3 Generation, Isolation and characterization of mouse BM-DCs

Mouse DC subsets can be generated *in vitro* from Flt3-L-supplemented BM culture. Freshly isolated BM cells cultured in Flt3-L-containing medium induced the generation of $CD11c^{+}B220^{+}$ PDC and $CD11c^{+}B220^{-}$ MDC subsets. According to the published data , mouse BM culture cells were harvested and isolated on day 7 which is the peak of the culture, 15-20% of the cells were $CD11c^{+}B220^{+}$ (PDC) cells whereas the remaining $CD11c^{+}B220^{-}$ (MDC) cells (Figure 4.5). Both DC subsets have an immature phenotype and were used for mRNA expression study.

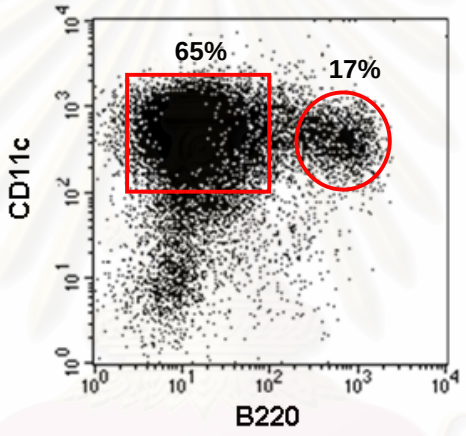


Figure 4.5 : *In vitro* generation of mouse BM-derived DC subsets. BM cells were cultured in medium supplemented with mouse Flt3-L. CD11c-FITC vs B220-PE FACS analysis after 7 days culture is shown. An electronic gate was determined on $CD11c^{+}$ cells and B220 expression was analyzed ($^{+}$ = PDC, $^{-}$ = MDC). Results shown are representative of three experiments.

4.2 COMPARISON OF TLR EXPRESSION ON DC SUBSETS AND OTHER APCS FROM: MACAQUE, HUMAN AND MOUSE

We investigated the expression of the following TLRs: TLR3, 4, 7, 8, and 9 using RT-PCR on macaque DC populations and compared this to human and murine DC subsets. To avoid contamination from other cell types, DC subsets and other APCs were sorted by flow cytometry (FACS) (the purity is >95%). As can be seen in Figure 4.6, macaque DC populations clearly differ in their TLR expression patterns.

Transcripts of TLR3 and TLR4 were detected in MDCs and mo-DCs, whereas TLR7 was only expressed in PDCs and MDCs but not in mo-DCs. TLR8 mRNA was equally expressed in all DC subsets. TLR9 was exclusively expressed in PDCs.

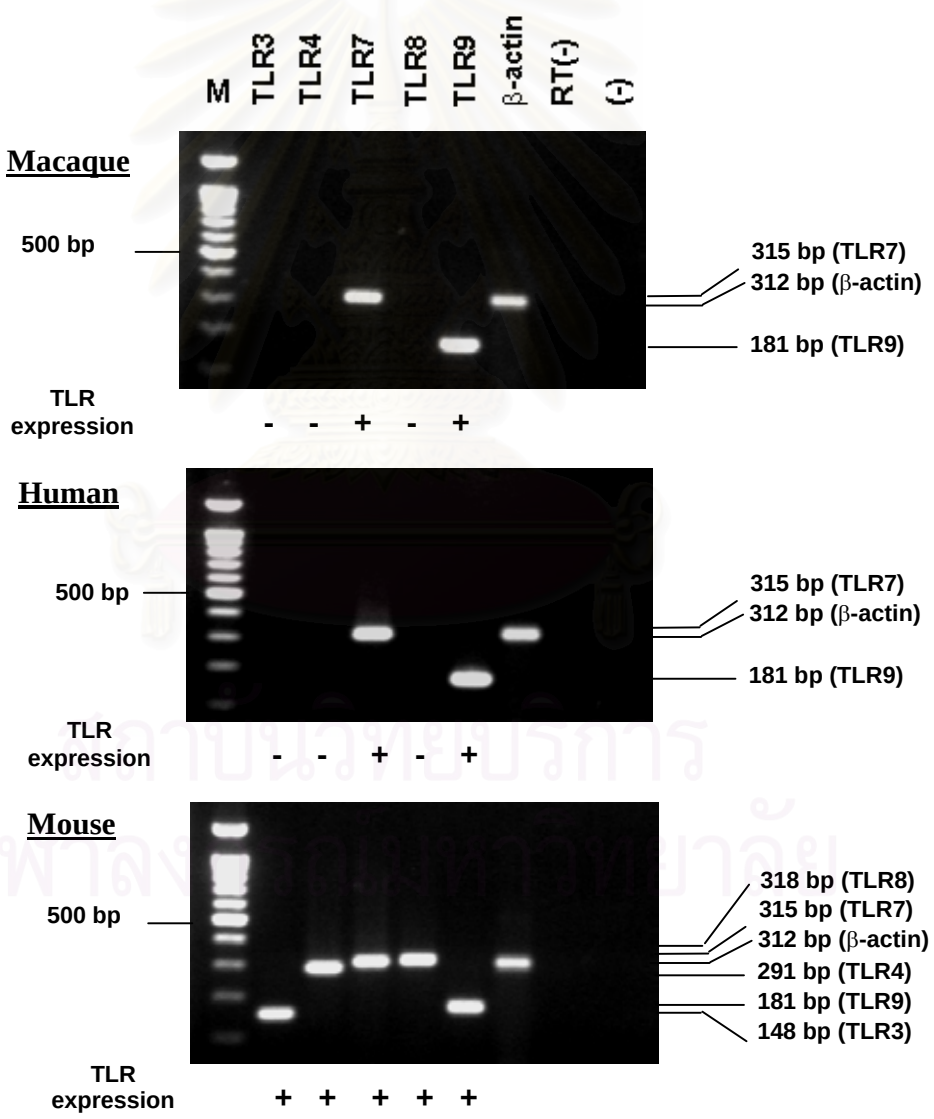
Comparison of the TLR expression in DC subsets from different species (Figure 4.6 and Table VII) revealed that macaque and human DCs express similar sets of TLRs in contrast to mouse DCs. In mouse, both PDCs and MDCs express TLR9, whereas only macaque and human PDCs but not MDCs express this TLR. In addition, macaque and human PDCs do not express TLR3, TLR4 and TLR8 that are highly expressed in murine PDCs.

Furthermore, TLR expression on other APCs from rhesus macaques were also investigated and compared to that of human APCs, as for DC subsets, similar TLR expression patterns were observed on both macaque and human monocytes and B cells. Transcripts of TLR4, TLR7 and TLR8 were detected in both monocytes and B cells, whereas TLR9 was expressed only in B cells.

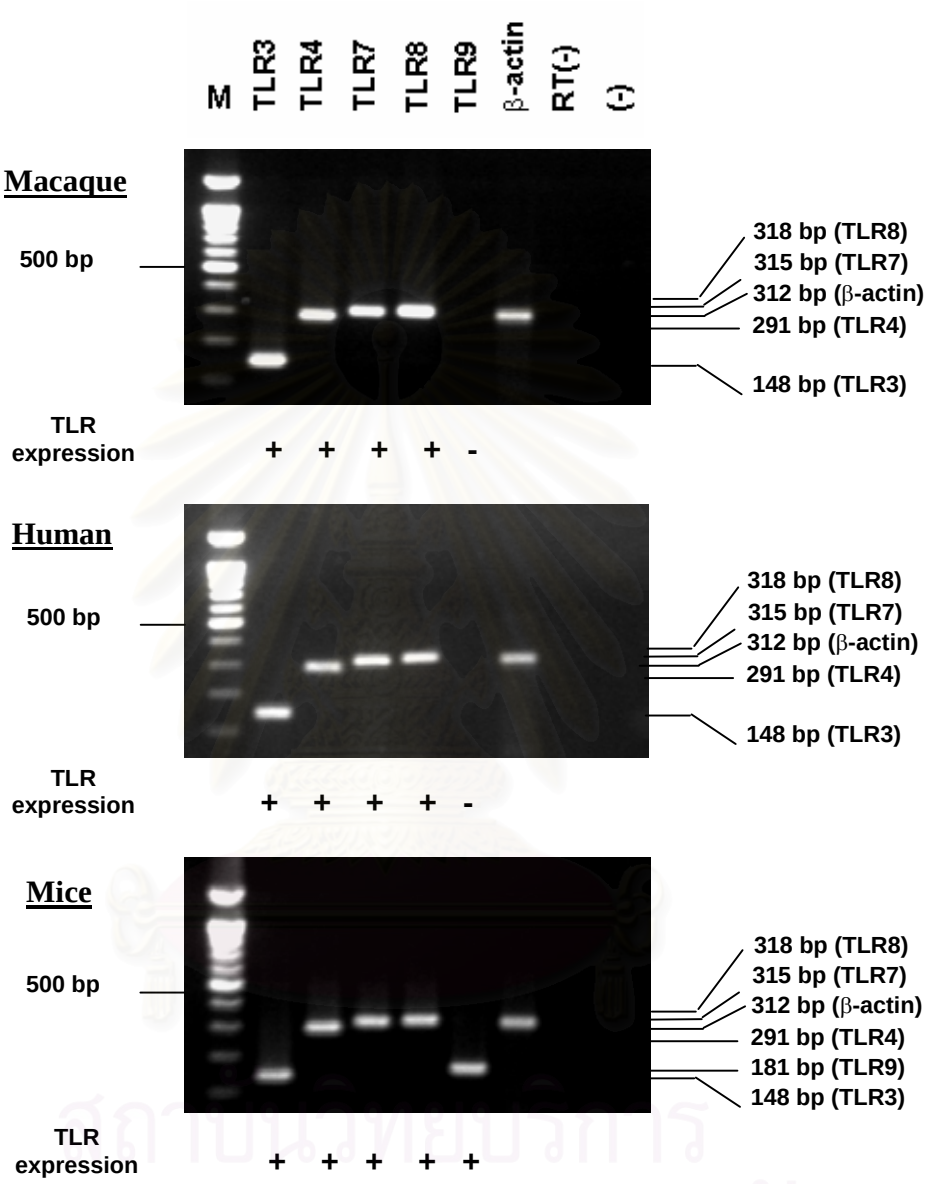
These results indicated that macaques APC subsets including PDCs, MDCs, mo-DCs, monocytes and B cells expressed a similar set of TLRs as human APC subsets.

Figure 4.6 : mRNA expression of TLRs in APCs from different species.
mRNA expression of TLR 3, 4, 7, 8, and 9 was examined in purified PDCs (a), MDCs (b), mo-DCs (c), monocytes (d), and B cells (e) by RT-PCR. For detection of mRNA expression, one set of primers was designed for using with three species. The data shown are representative of three independent experiments. β-actin as positive control, RT(-) as non-reverse transcribed RNA, (-) as negative control and M as a marker.

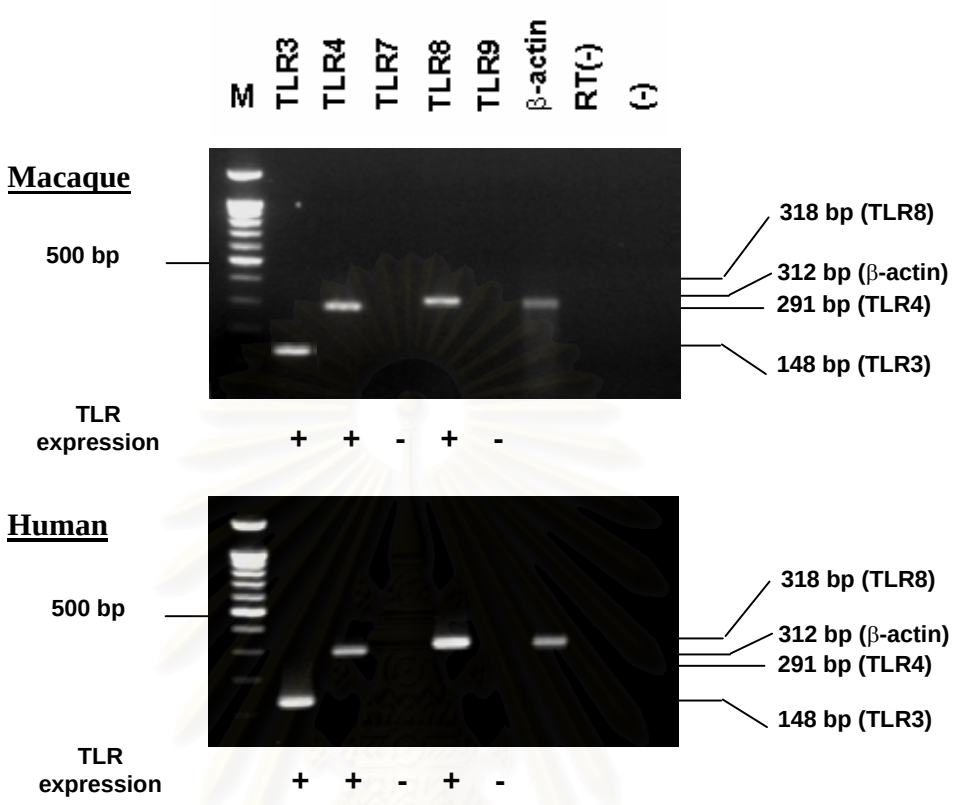
a : Plasmacytoid dendritic cells



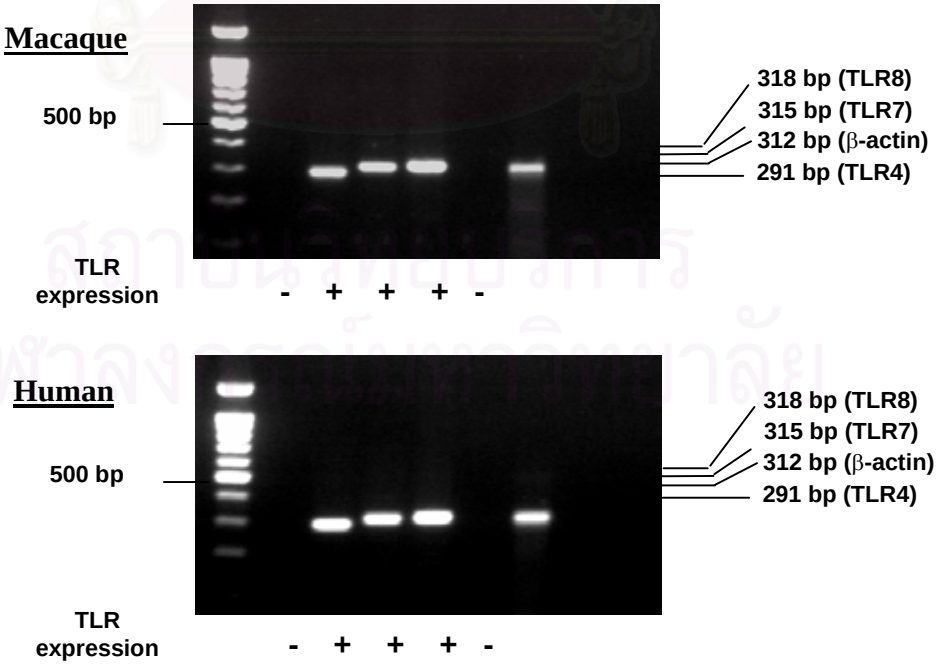
b : Myeloid dendritic cells



c : Monocyte-derived DCs



d : Monocytes



e : B cells

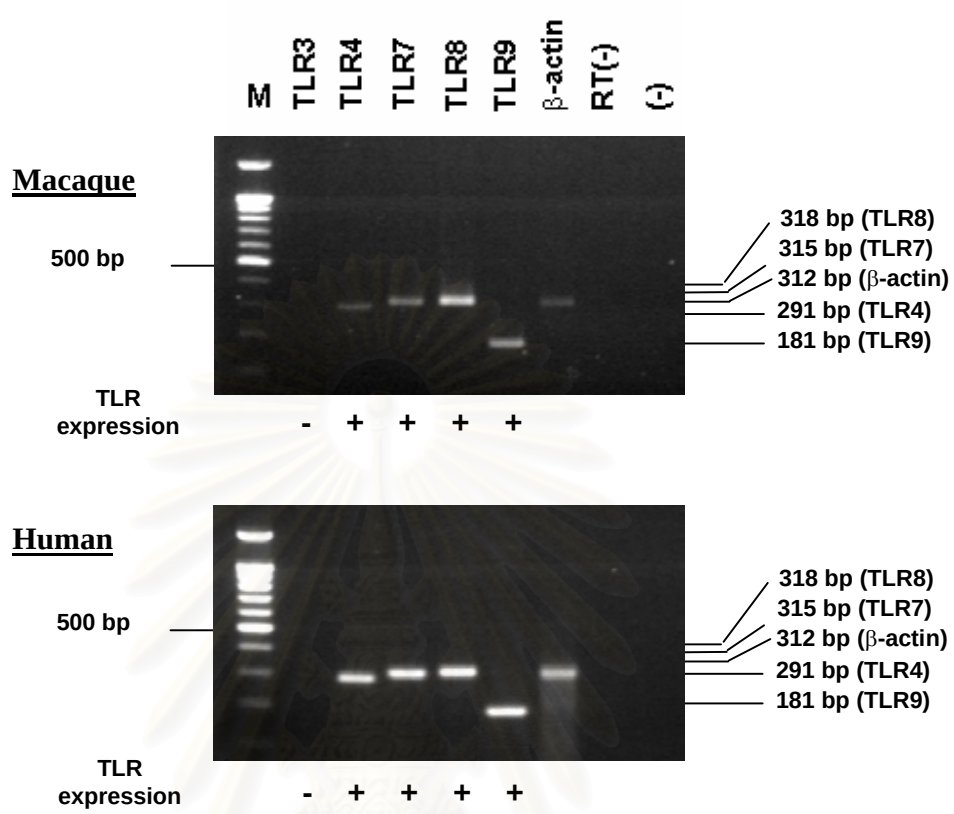


Table VII : Comparison of TLR expressions in DCs (between macaque, human and mouse) and other APCs (between macaque and human)

Type of cells Species	**TLR3**	**TLR4**	**TLR7**	**TLR8**	**TLR9**
Plasmacytoid DCs					
Macaque	-[a]	-	+	-	+
Human	-	-	+	-	+
Mouse	+[b]	+	+	+	+
Myeloid DCs					
Macaque	+	+	+	+	-
Human	+	+	+	+	-
Mouse	+	+	+	+	+
Monocyte-derived DCs					
Macaque	+	+	-	+	-
Human	+	+	-	+	-
Monocytes					
Macaque	-	+	+	+	-
Human	-	+	+	+	-
B cells					
Macaque	-	+	+	+	+
Human	-	+	+	+	+

a indicate the absence of mRNA expression of each TLRs
b indicate the presence of mRNA expression of each TLRs

4.3 TLR FUNCTION IN MACAQUE APCS

4.3.1 Effects of TLR ligation on phenotypic differentiation

Since macaque and human DCs subsets and other APCs express a similar set of TLRs, we next compared the function of these TLRs. First, we characterized the phenotypic differentiation of macaque PDCs, MDCs, mo-DCs, monocytes and B cells after being stimulated *in vitro* with different TLR ligands for 24 hours. The concentration of TLR ligands used was optimized following titrations experiments.

4.3.1.1 Plasmacytoid dendritic cells (PDCs)

Consistent with their expression of TLR7 and 9, PDCs differentiated in response to imiquimod, a ligand for TLR7, and CpG ODN, a ligand for TLR9, as shown by an up-regulation of CD86 expression (Figure 4.7). PDCs stimulated with poly(I:C), E.Coli LPS, or ssPolyU/LyoVec, ligands for TLR3, TLR4, or TLR8, respectively, did not showed any up-regulation of CD86 expression.

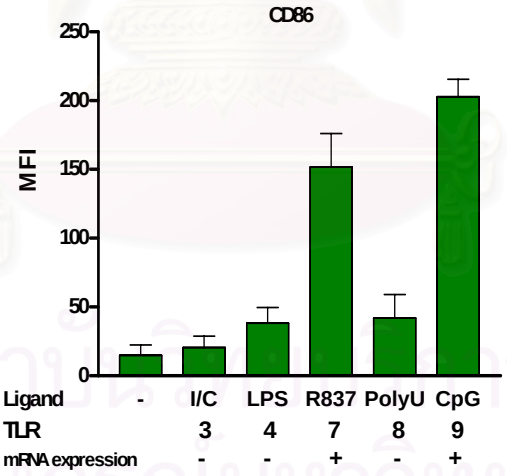


Figure 4.7 : Phenotypic changes in macaque PDCs in response to TLR ligation. Purified PDCs were incubated with TLR3 ligand (poly (I:C); 100 μg/ml), TLR4 ligand (LPS; 5 μg/ml), TLR7 ligand (imiquimod or R837; 3 μg/ml), TLR8 ligand (SSpolyU; 5 μg/ml), and TLR9 ligand (CpG 2006; 5 μg/ml). Mean fluorescence intensity (MFI) of CD86 was analyzed by flow cytometry. Data is shown as means ± SD (n=3).

4.3.1.2 Myeloid dendritic cells (MDCs)

In contrast to PDCs, MDCs upregulated the costimulatory molecules of CD86 and CD40 when stimulated with poly(I:C), E.Coli LPS, imiquimod and ssPolyU/LyoVec, ligands for TLR3, TLR4, TLR7 and TLR8, respectively, correlating with their expression of TLR3, TLR4, TLR7 and TLR8 (Figure 4.8). Consistent with their lack of TLR9 expression, MDCs did not respond to CpG ODN.

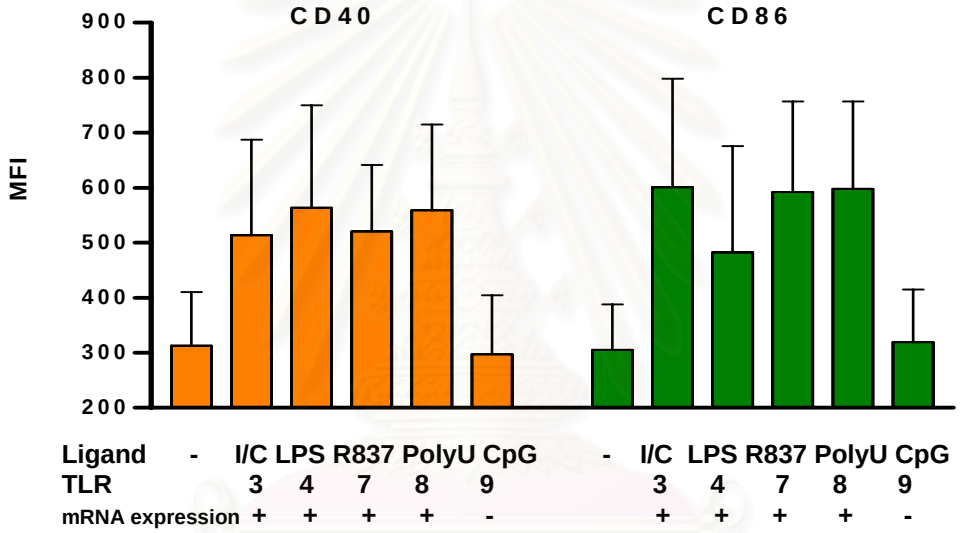
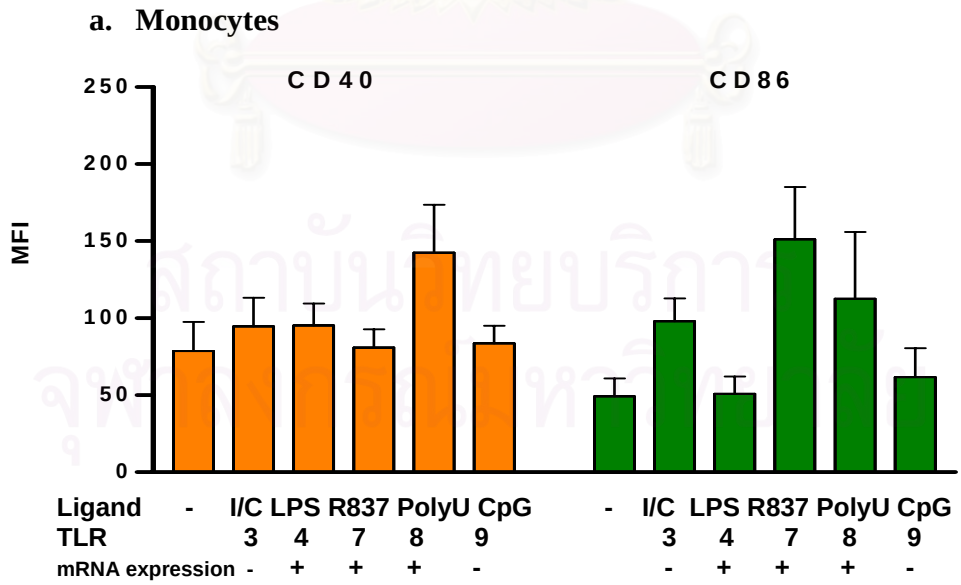


Figure 4.8 : Phenotypic changes in macaque MDCs in response to TLR ligation. Purified MDCs were incubated with TLR3 ligand (poly (I:C); 100 μg/ml), TLR4 ligand (LPS; 5 μg/ml), TLR7 ligand (imiquimod or R837; 3 μg/ml), TLR8 ligand (SSpolyU; 5 μg/ml), and TLR9 ligand (CpG 2006; 5 μg/ml). Mean fluorescence intensity (MFI) of CD40 and CD86 was analyzed by flow cytometry. Data is shown as means ± SD (n=3).

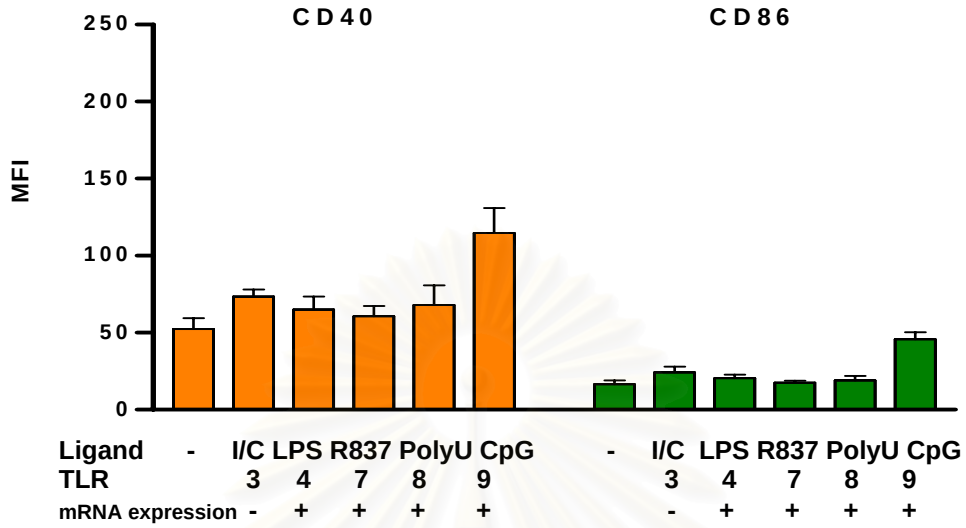
3.1.3 Other antigen presenting cells (APCs) : Monocytes and B cells

Neither macaque monocytes nor B cells showed dramatic phenotypic differentiation when exposed to the all TLR ligands. However, in response to poly(I:C), imiquimod and ssPolyU/LyoVec, monocytes did show a small upregulation of CD86 and CD40. In contrast, in response to E.coli LPS, monocytes showed upregulation of CD40 but no changes in CD86 (Figure 4.9a). B cells showed a slight up-regulation of CD86 and CD40 in response to CpG ODN only (Figure 4.9b). However, when compared with the responses in PDCs, these effects were minimal.

Figure 4.9 : Phenotypic changes in macaque Monocytes and B cells in response to TLR ligation. Purified monocytes and B cells were incubated with TLR3 ligand (poly (I:C); 100 μg/ml), TLR4 ligand (LPS; 5 μg/ml), TLR7 ligand (imiquimod or R837; 3 μg/ml), TLR8 ligand (SSpolyU; 5 μg/ml), and TLR9 ligand (CpG 2006; 5 μg/ml). Mean fluorescence intensity (MFI) of CD40 and CD86 was analyzed by flow cytometry. Data is shown as means ± SD (n=3).



b. B cells



3.2 Effects of TLR ligation on cytokine production

Subsets of DCs have been reported to have distinct patterns of cytokine production. In this regard, PDCs may be the only APCs capable of producing high levels of interferon type I (IFN-α) [161]. Therefore, we further determine the function of TLRs by investigating the secretion of IFN-α in PDCs, IL-6 in B cells and TNF-α and IL-12p70 by the other APCs in response to TLR3, 4, 7, 8 and 9 ligands.

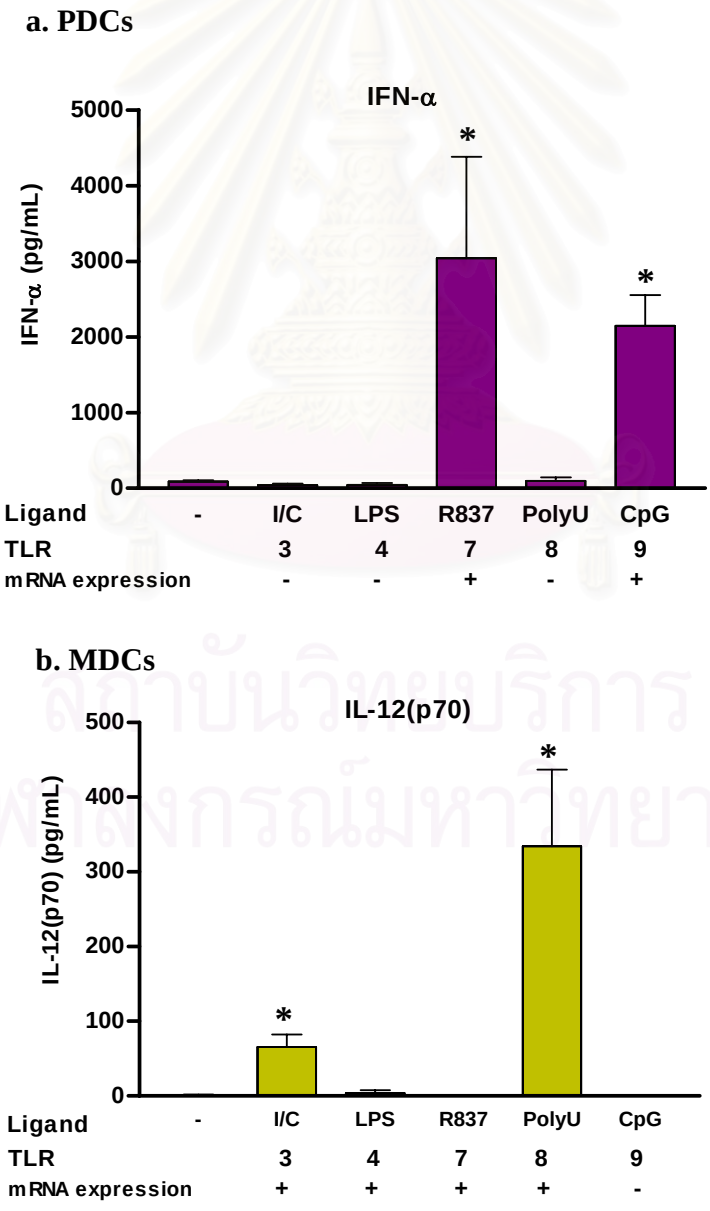
Macaque PDCs produced remarkably high level of IFN-α (880-5,500 pg/mL) in response to imiquimod and CpG ODN (n=3, $p \leq 0.05$ compared with unstimulated PDCs) (Figure 4.10a). However, these amounts were lower as compared to levels reported to be produced by human PDCs (30,000-50,000 pg/mL) by our group [17]. Undetectable or very low amounts of IFN-α were found in supernatants of PDCs incubated with other TLR ligands. Thus, these data confirm that IFN-α is induced only by TLR7 and 9 ligand-exposure of PDCs, which is consistent with their TLR7 and 9 expression.

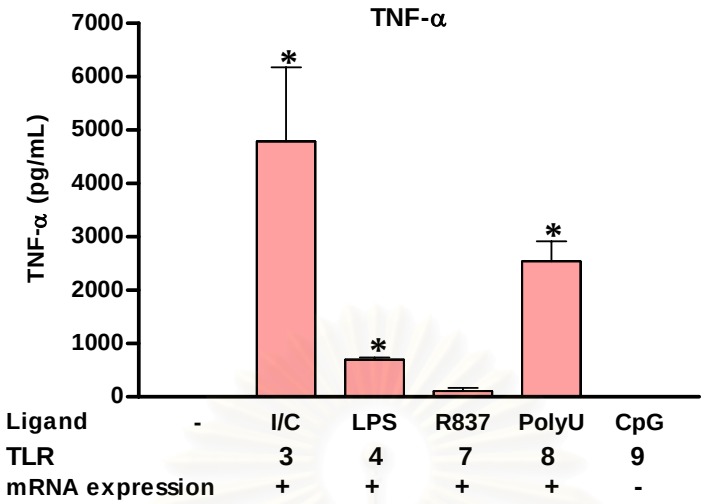
Significantly increased levels of IL-12(p70) (40-510 U/mL) were observed when macaque MDCs were stimulated with poly(I:C) and ssPolyU/LyoVec (n=3, $p \leq 0.05$ compared with unstimulated MDCs, respectively) whereas when MDCs were stimulated with E.coli LPS, imiquimod and CpG ODN showed no detectable level of IL-12p70 (Figure 4.11b). Of note, macaque MDCs produced high levels of TNF-α (765-7,026 pg/mL) in response to poly(I:C), LPS and ssPolyU/LyoVec (n=3, $p \leq 0.05$ compared with unstimulated MDCs) (Figure 4.11b). Imiquimod induced low but detectable levels of TNF-α (22-213 pg/mL, n=3) in MDCs.

Macaque monocytes produced very high levels of TNF-α (344-3,958 pg/mL) in response to E.Coli LPS and ssPolyU/LyoVec (n=3, $p \leq 0.05$ compared with unstimulated monocytes) (Figure 4.11c). Macaque B cells stimulated with E.Coli LPS, imiquimod, ssPolyU/LyoVec, and CpG ODN induced high levels of IL-6 (240-818 pg/mL) compared with unstimulated B cells (n=3, $p \leq 0.05$) (Figure 4.11d).

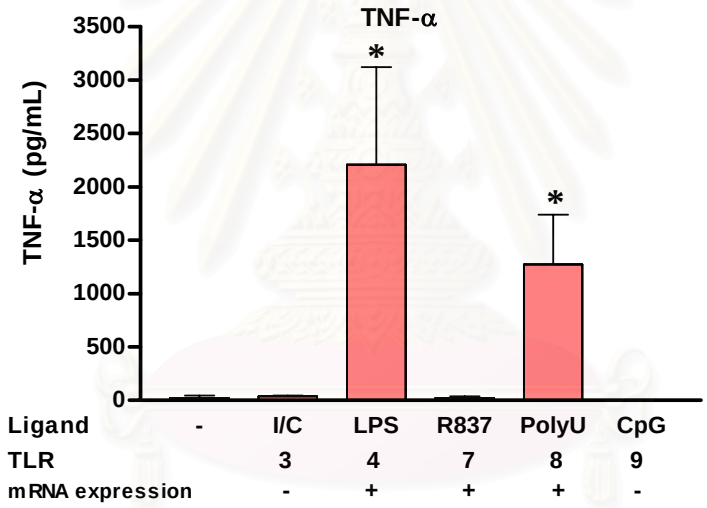
Figure 4.10 : Cytokine production by macaque APCs in response to different TLR ligands.

APCs were stimulated with TLR3 ligand (poly (I:C); 100 μg/ml), TLR4 ligand (LPS; 5 μg/ml), TLR7 ligand (imiquimod or R837; 3 μg/ml), TLR8 ligand (SSpolyU; 5 μg/ml), and TLR9 ligand (CpG 2006; 5 μg/ml) for 24 h. Supernatants of APCs cultures were measured for different cytokines by ELISA:(a) PDCs; IFN-α, (b) MDCs; IL-12p70 and TNF-α, (c) monocytes; TNF-α, and (d) B cells; IL-6. Data is shown as means ± SD (n = 3 for each groups; *, $p \leq 0.05$, compared with unstimulated cells, paired student's *t* test).

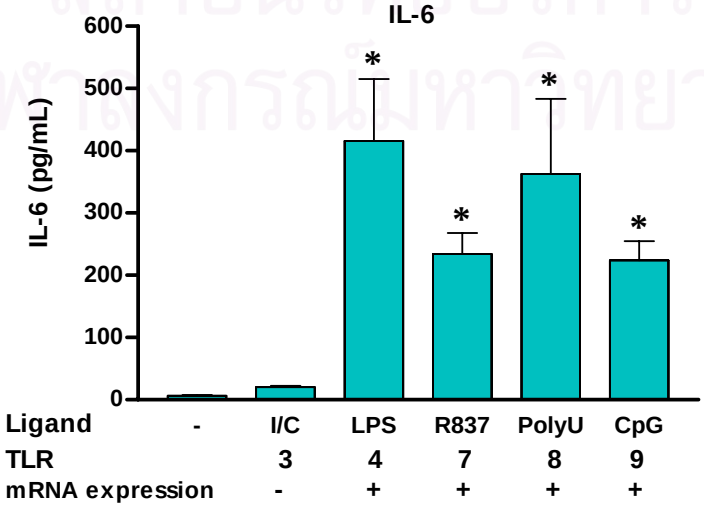




c. Monocytes



d. B cells



4.4 MONOCYTE-DERIVED DENDRITIC CELLS (MO-DCS) COMPARISON BETWEEN MACAQUE AND HUMAN

For macaque mo-DCs, treatment with poly(I:C), E.Coli LPS and ssPolyU/LyoVec resulted in dramatically increased maturation, reflected by up-regulation of CD86, CD83 and CD40. However, compatible with their lack of TLR7 and TLR9 expression, imiquimod and CpG ODN did not induce any maturation. In comparison, human mo-DCs were also activated by the same TLR ligands (although they responded to ssPolyU/LyoVec to a lesser degree) (Figure 4.11).

Human mo-DCs produced high levels of IL-12p70 (73-144 U/mL, n=2) in response to poly(I:C), E.Coli LPS, or ssPolyU/LyoVec (Figure 4.12). In contrast, macaque mo-DCs did not produce IL-12p70 in any condition. However, CD40L stimulation can trigger IL-12p70 production (20-45 U/mL, n=2) in macaque mo-DCs, indicating that these cells are capable of production of this cytokine. Macaque mo-DCs produced high levels of TNF-α (356-1730 pg/mL) in response to E.Coli LPS and ssPolyU/LyoVec (n=3, $p \leq 0.05$ compared with unstimulated macaque mo-DCs) and produced low levels of TNF-α (147-168 pg/mL) in response to poly(I:C). Interestingly, high levels of IFN-α production were induced by poly(I:C), a ligand for TLR3, in both macaque (115-371 pg/mL, n=3) and human mo-DCs (560-1132 pg/mL, n=2).

Figure 4.11 : Comparison of phenotypic changes in macaque and human mo-DCs (*in vitro*) in response to different TLR ligands for 24 h. Purified macaque and human mo-DCs (n = 3 and n = 2, respectively) were stimulated with different TLR ligands. Mean fluorescence intensity (MFI) values of CD40 (a), CD83 (b), or CD86 (c) was analyzed by flow cytometry. Data is shown as means ± SD for macaque mo-DCs and means for human mo-DCs.

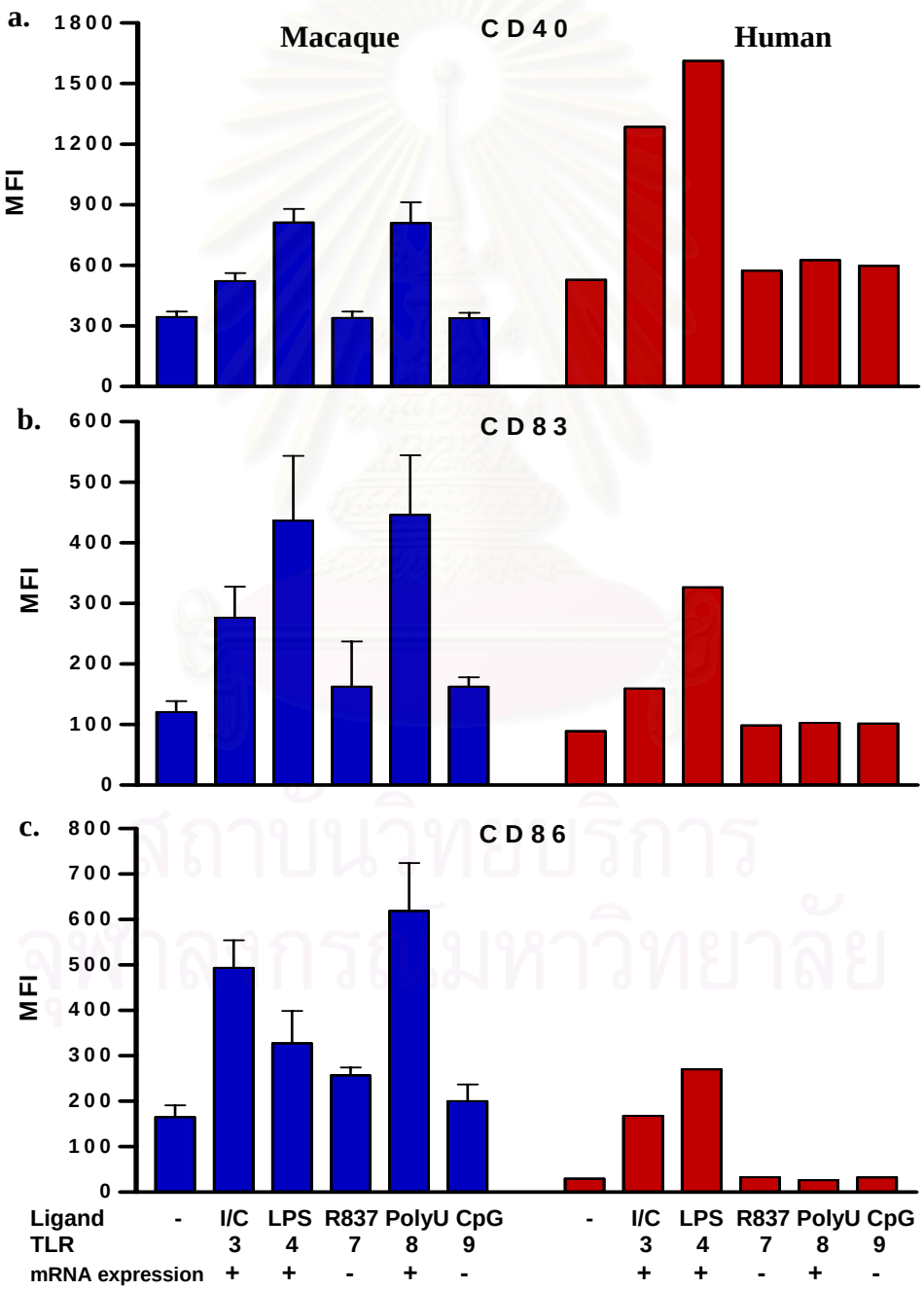
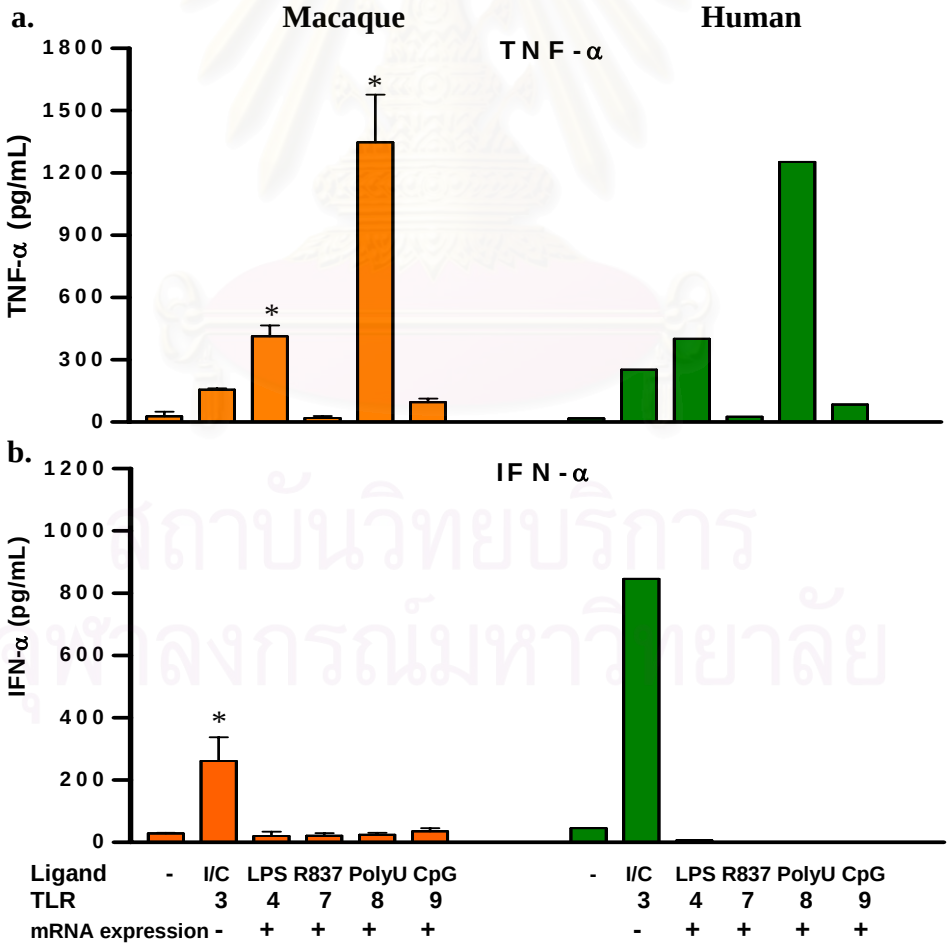
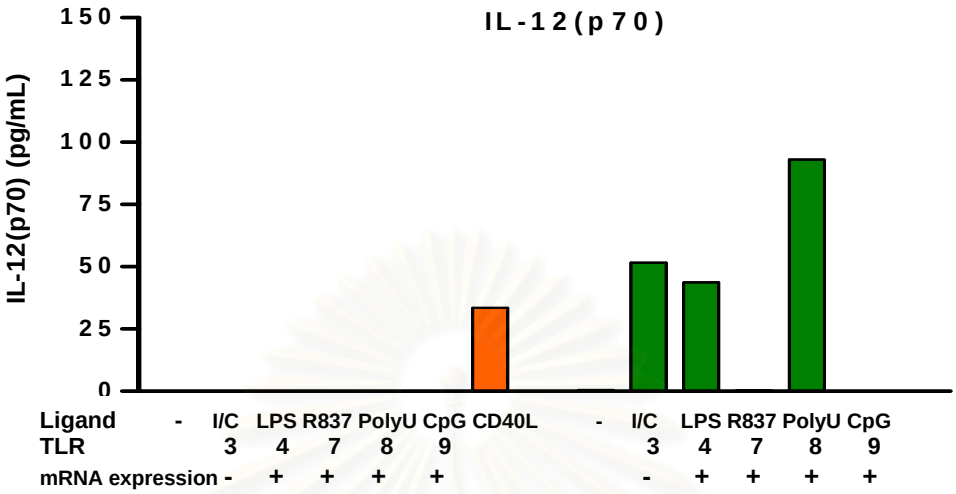


Figure 4.12 : Comparison of cytokine production in macaque and human mo-DCs (*in vitro*) in response to different TLR ligands for 24 h. Cytokine production of TNF-α (a), IFN-α (b) and IL-12p70 (c) were measured in the supernatant by ELISA, after exposure of macaque or human mo-DCs (n = 3 and 2, respectively) to TLR3 ligand (poly (I:C); 100 μg/ml), TLR4 ligand (LPS; 5 μg/ml), TLR7 ligand (imiquimod; 3 μg/ml), TLR8 ligand (SSpolyU; 5 μg/ml), and TLR9 ligand (CpG 2006; 5 μg/ml). Of note, CD40L fibroblast cell line was additionally used to stimulate macaque mo-DCs (n=2). The J558L transfected with CD40L fibroblast cell line was additionally used to stimulate macaque mo-DCs. Data is shown as means ± SD for macaque mo-DCs) (n = 3, * $p \leq 0.05$, compared with unstimulated cells, paired student's *t* test) and shown as means for human mo-DCs (n = 2).



c.



CHAPTER V

DISCUSSION AND CONCLUSION

A careful selection of animal models to evaluate strategies for vaccination and the selection of vaccine components, such as adjuvants, for clinical use, is important. It remains possible that animals have different responses to particular adjuvants than humans. In the vaccine development field, there has been a recent interest in using TLR ligands as adjuvants; in this regard, it should be noted that there are major differences in animal models, especially in TLR expression patterns between mouse and human [4, 162]. We hypothesized that rhesus macaques might be a better animal model for evaluating TLR adjuvant vaccine. However, studies on TLR expression and function in these animals were lacking. Therefore, *ex vivo* experiments on freshly isolated macaque APCs were performed in this study for assessing TLR expression and functionality.

In this study, two techniques were used to isolate DC subsets depending on the subsequent characterization of the cells. Based on the high purity of isolated cells (>95%), FACS technique was used to isolate the cells for mRNA expression studies. This minimized contamination with mRNA from other cell types. However, a limitation of cell sorting using FACS is the pre-activation of isolated cells during the process, partly due to isolation at room temperature and to mechanical forces that are applied on the cells. Hense, for functional study MACS technique was used to isolate the cells instead. By MACS, the pre-activation of isolated cells was prevented because of all sorting steps were performed on ice or using ice-cold buffers A disadvantage of the MACS technique is the lower cell purity obtained (>80%), as compared to isolation by FACS. In order to improve the purity of isolated cells, further experiments have to be performed such as to perform negative selection to deplete the contaminating cell populations (for DCs subsets, these are mainly B cells and monocytes) or use more consecutive columns to increase the

purity. Nevertheless, it should be realized that increasing the number of columns will lead to a decreasing yield of isolated cells. Together with our observation that macaques have lower amounts of circulating DCs as compared to humans and with the fact that smaller volumes of blood can be obtained from macaques due to their smaller size and weight, increasing the purity by using more consecutive columns was not feasible in this study, restricting cell purities to >80% only.

In a previous study, it was shown that DC subsets in rhesus macaque closely resemble humans DCs, including expression of DC markers [16]. Therefore, rhesus macaque DC subsets can be characterized and isolated using anti-human mAbs that cross-react with rhesus. For isolation of macaque DCs, we observed that indeed macaque PDCs could be identified as HLA-$DR^{+}CD123^{+}$ cells similar to human PDCs. However, macaque PDCs showed substantially differences in their CD123 staining. In comparison to humans, few HLA-$DR^{-}CD123^{+}$ cells were observed in macaque. As a result of this observation, we initiated a new method for sorting macaque PDCs by using anti-PE microbeads to sort CD123-PE stained macaque PDCs directly from PBMCs with a purity of >80%. Furthermore, we also found that antibodies to CD11c (clone 3.9, Biosource, CA), a myeloid marker, did not cross-react with macaque MDCs in our hands. In constrast to the previous study showing that this mo-Ab (clone 3.9) cross-reacted with macaque [61] as well as the species specificity recommended from the commercial supplier. Instead, we used antibodies BDCA-1 (CD1c) for MDCs isolation. In addition, we generated a modified method for obtaining macaque mo-DCs from blood monocytes in this study by using similar amounts of GM-CSF (100 ng/ml), but 10 times lower amounts of IL-4 and only 1% FCS. By our method, the higher yield of immature macaque mo-DCs was obtained comparing to the original method [59].

To study TLR expression patterns on APCs in human, rhesus macaque and mice, we generated the new primer sets (TLR3, 4, 7, 8, 9 and β-actin) that are specific for all three species. In consequence, our study is the first comparative cross-species study on TLR expression patterns (TLR3, 4, 7, 8 and 9) of APCs in

human, rhesus macaque and mice. Our detailed analysis showed that blood DC subsets of rhesus macaque expressed the same sets of TLRs as those of human but were substantially different from mouse DC subsets. In macaque and human, MDCs expressed TLR3, 4, 7 and 8 whereas PDCs expressed only TLR7 and 9, in contrast to mouse in which both DC subsets expressed all TLRs (TLR3, 4, 7, 8 and 9). Additionally, TLR expression patterns in macaque mo-DCs (i.e., TLR3, 4 and 8), monocytes (i.e., TLR4, 7, and 8) and B cells (i.e., TLR4, 7, 8, and 9) were also similar to human.

For analysis of TLR mRNA expression in detail, two controversial topics have been reported in humans. The first is whether human MDCs and monocytes express TLR7 [10-13, 137] and the second is whether human B cells express TLR4 and TLR8 [10, 141, 142, 163]. Concerning TLR7, our results clearly demonstrated that mRNA expression in both macaque and human MDCs and monocytes. Moreover, we observed that both macaque and human B cells expressed TLR4 and TLR8. There are several possibilities that may account for these observations. The purity of the isolated cells should be the most important issue. Taking this into consideration, >95% purity of the isolated cells (as determined by FACS analysis) was obtained from our study and also from the other studies in human [10-13, 137, 141, 142]. The mRNA from the remaining contaminated cells might be amplied and responsible for the results obtained. However, this possibility was ruled out in our study, because PCR sensitivity testing showed that the minority of the contaminated cells (less than 5,000 cells per one PCR reaction) could not be amplified by our method. Still, further experiments need to be performed by using the known proportion of contaminated cells for testing the detection limit of our method. The second issue for this discrepancy might be the primer design. The three studies in human MDCs and monocytes showed different observations of TLR7 expression and used different TLR7 primer sets [11, 13]. By using the RT-PCR method, the study from Krug et al. [11] observed TLR7 expression whereas Jorossay et al. [13] cound not detect TLR7 expression in both human MDCs and monocytes. Kadowaki et al. [12] found weak TLR7 expression by RT-PCR, but significant expression of

TLR7 by real-time PCR in both human cell types. According to the observation by Kadowaki et. al. [12], it seems reasonable to suppose that not only the different primer sets but also the different assay used should be taken into consideration for interpretation of the results; in their hands, real-time PCR was more sensitive than conventional PCR for detection of mRNA expression [12]. However, in our study, we chose to perform only conventional RT-PCR because our main interest was to determine which genes are expressed and not to quantify gene expression.

In functional studies, it should be realized that our study has the limitation in the purity of isolated cells, as mentioned above. By using MACS separation in our study, only > 80% purity was obtained. Therefore, contaminating cells could be responsible for the observed results especially for the cytokine production. In order to confirm our observations, further experiments need to be performed such as increasing the purity of the isolated cells and/or using more specific technique such as intracellular cytokine staining (ICS) to identify which individual cells are responsible for the observed cytokine production.

While this study showed that TLR expression patterns in macaques APCs generally mirrored that of human APCs, the responsiveness of macaques APCs to certain activation ligands partially differed from that of human APCs. Interesting, unlike human mo-DCs, macaque mo-DCs did not produce bioactive IL-12p70 in response to any of the tested TLR ligands (TLR3, 4, 7, 8 and 9 ligands). It is well known that the biologically active form of IL-12p70 is a heterodimeric cytokine, composed of a p35 subunit and a p40 subunit [164]. Therefore, the production of bioactive IL-12p70 requires the coordination of two subunits protein that are encoded by different genes. Hence, further experiments need to be performed such as to study IL-12p40 cytokine production, which is representative for IL-12 transcriptional regulation, by ELISA or on mRNA level. In addition, a number of studies have been reported that activated T cells, either by CD40-CD40L interactions or through co-stimulation of T cell-derived IFN-γ [11, 164], also increase IL-12p70 production by DCs. To test this concept on macaque mo-DCs,

we performed a co-culture of these cells with CD40L transfected cells to mimick intereaction with activated T cells [159]. We indeed observed IL-12p70 production; however, the amount produced was very low, similar to another study using macaque mo-DCs [59]. Thus macaque mo-DCs are capable of secreting IL-12p70 in macaque mo-DCs and this requires CD40L costimulation in addition to TLR activation, in contrast to human mo-DCs.

As mentioned earlier, we found that TLR7 is expressed in both macaque and human MDCs and monocytes. However, we observed that TLR7 stimulation using the TLR7 ligand, imiquimod, of macaque MDCs and monocytes resulted in low to undetectable levels of IL-12p70 and TNF-α production, respectively. This finding corresponded with studies showing that human MDCs produced low levels of IL-12p70 in respond to imiquimod (TLR7 ligand) [165] and human monocytes did not produced TNF-α in respond to loxoribine (TLR7 ligand) [166]. However, both of those studies showed in addition that human MDCs and monocyte can produce high level of IL-12p70 and TNF-α when stimulated with the alternative TLR7 ligand resiquimod (R-848), which has been reported that to be a more potent stimulus than imiquimod [165, 166]. Thus, in our study, a possible explanation for the lack of cytokine production by macaque MDCs and monocytes might be that imiquimod and loxorubine are not potent enough for NF-kB activation in these macaque cells. Alternatively, it might be that TLR7 on macaque MDCs and monocytes is not functional. Further experiments using the potent TLR7 ligand R848 should be performed to answer these issues.

In literature, there is no information on TLR expression and the activity of their ligands in macaque B cells, in contrast to human and mice. Although it is well established that murine B cells respond to the TLR4 ligand LPS [167], TLR4 expression in human B cells was found to be weak [10, 163] and B cells were unresponsive to LPS [163]. However, our study demonstrated that both human and macaque B cells express TLR4 and macaque B cells could response to LPS by producing the cytokine IL-6 cytokine. Furthermore, we observed the expression of

TLR8 in human and macaque B cells and could demonstrate that macaque B cells respond to TLR8 ligand. This is in line with the study by Bourke et al. showing that B cells express human TLR8 [142] and can activated by TLR8 ligand (Tomai et al. [168]). However, other reports have described lack of TLR8 mRNA expression and functional TLR8 in human B cells [165, 169, 170]. In order to prove the mRNA expression, the purity of isolated B cells, primer design and the method (such as RT-PCR and real-time PCR) should be taken into account as mentioned before. In order to convincingly demonstrate that TLR4 and TLR8 ligands can activate macaque B cells, further experiments should be performed to extend our results such as studying B cell proliferation and IgM production after TLR stimulation.

Based on our results, we can explain why some adjuvants induce potent immune response in mouse models but not in humans and non-human primates. Of note, while CpG ODN are potent adjuvants for induction of both humoral and cellular immunity in mice [143, 171, 172], they can induce only humoral immune responses in humans [150] and in non-human primates [173-175]. These differences are likely due to differential expression of TLR9, which is expressed on murine MDCs, but absent in human and macaque MDCs. There are other controversies in studies using different animal models for testing vaccine adjuvants. Studies in mice have shown that CpG ODN is more effective than TLR7/8 agonists as vaccine adjuvants [20]. In contrast, subsequent primate studies by the same group demonstrated that a vaccine with TLR7/8 agonists dramatically enhanced the magnitude and improved the quality of Th1 and $CD8^+$ T cell responses, compared to animals immunized with vaccine containing CpG ODN [176]. Our results indicate that TLR7 and TLR8 are broadly expressed on all APCs in both humans and macaques, whereas TLR9 is limited to B cells and PDCs. Therefore, TLR7/8 agonists could indeed be more effective in non-human primates than CpG ODN for eliciting broad humoral and cellular immune responses, by targeting different sets of APCs. From our study, we can conclude that after TLR ligation, macaque APCs induced an innate immune response by up-regulation of costimulatory molecules and production pro-inflammatory cytokines. However, whether the effect of TLR

ligation can subsequently activate the adaptive immune response should be investigated. Further studies should be focused on the effect of TLR ligation both *in vitro* and *in vivo* for T cell stimulation studies.

In conclusion, our studies will open new avenues in the field of TLR research since we show that non-human primates share expression and to a certain level also functionality of TLR with humans. In addition, the immunization concept of broadly targeting APCs, and in particular DC subsets based on their specific TLR expression, could emphasize the importance of using non-human primates for the design of vaccines in which such responses might be important in humans. Our study provides important information for the rational design of animal models for evaluating the safety and activity of new vaccine adjuvants *in vivo.*

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APPENDICES

APPENDIX I

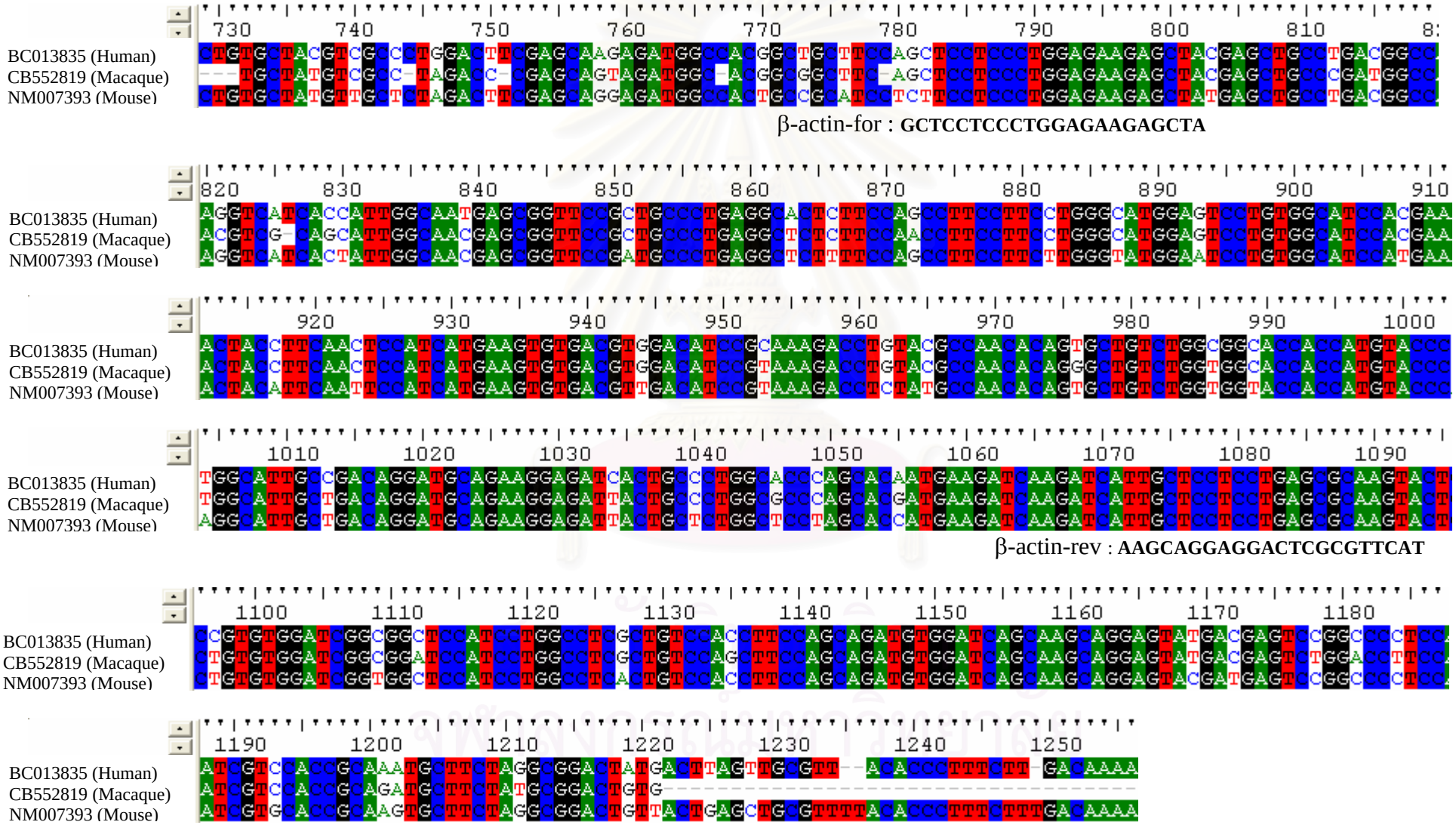
<u>Part I</u> : Sequence alignment and primer design for this study

Table VIII : Summaries of primer sequence used for RT-PCR and PCR optimal conditions for three species

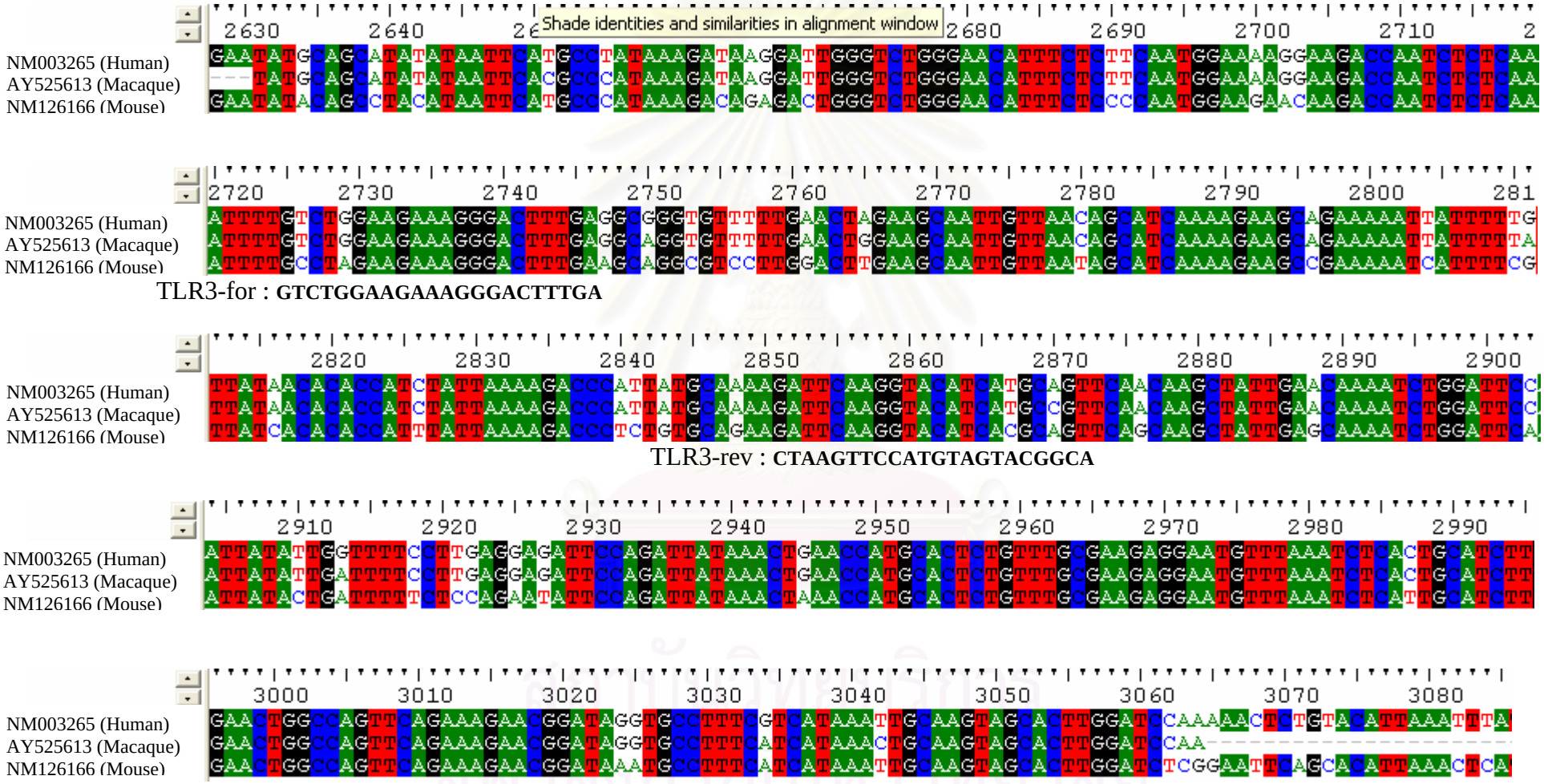
Oligo name	mRNA Accession Code*	Sequence (5'-3')	Expected length (bp)	Optimal Annealing Temp (°C)	Optimal Thermal Cycles
β-actin-for	BC013835[a]	GCTCCTCCCTGGAGAAGAGCTA	312	52	35
β-actin-rev	CB552819[b]	TACTTGCGCTCAGGAGGAGCAA			
	NM007393[c]				
TLR3-for	NM003265[a]	GTCTGGAAGAAAGGGACTTTGA	148	52	40
TLR3-rev	AY525613[b]	ACGGCATGATGTACCTTGAATC			
	NM126166[c]				
TLR4-for	NM003266[a]	TCAGCTCTGCCTTCACTACAGA	291	52	35
TLR4-rev	AY525614[b]	CTGTCCTCCCACTCCAGGTA			
	NM021297[c]				
TLR7-for	NM016562[a]	GACACTAAAGACCCAGCTGTGA	315	54	35
TLR7-rev	AY525617[b]	CTGGAGGAACTTGGACTTCTGA			
	AY035889[c]				
TLR8-for	AF245703[a]	CTGCGCTACCACCTTGAAGAGA	318	52	35
TLR8-rev	AY525618[b]	GTCAGGCCACTGGAGGATGGA			
	NM133212[c]				
TLR9-for	AF245704[a]	GCTGTTTGTGCTGGCCCACA	181	52	40
TLR9-rev	AY525619[b]	GAGGACACTCTGGCGGCAGA			
	NM031178[c]				

* GenBank, National Center for Biotechnology Information.
a *Homo sapiens* mRNA sequence (human)
b *Macaca mulatta* mRNA sequence (rhesus macaques)
c *Mus musculus* mRNA sequence (mouse)

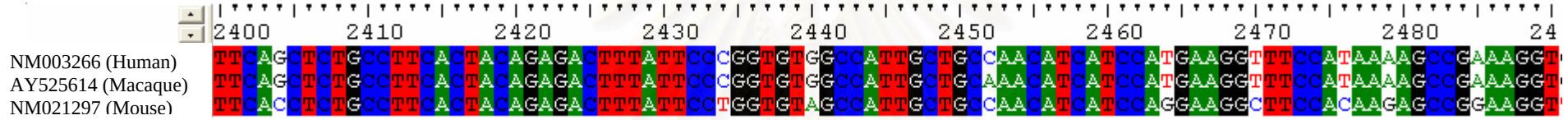
1. **Beta Actin :** Estimated product size = 312 bp



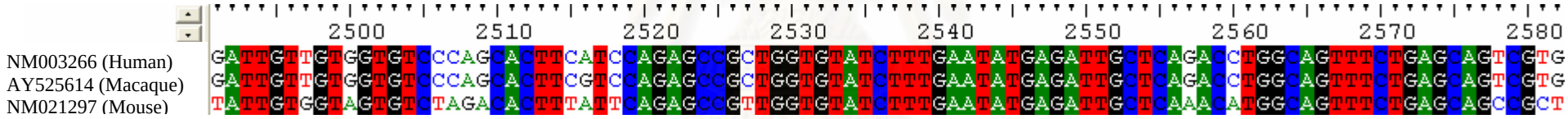
2. TLR3 : Estimated product size = 148 bp

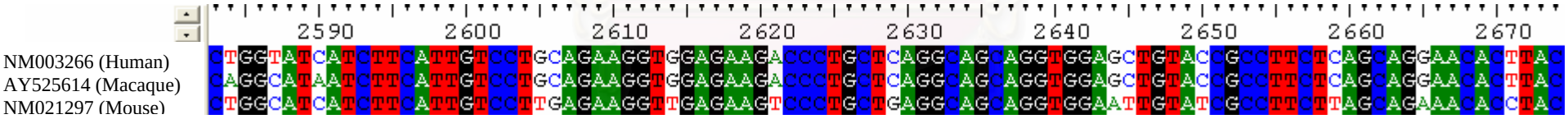


3. TLR4 : Estimated product size = 291 bp

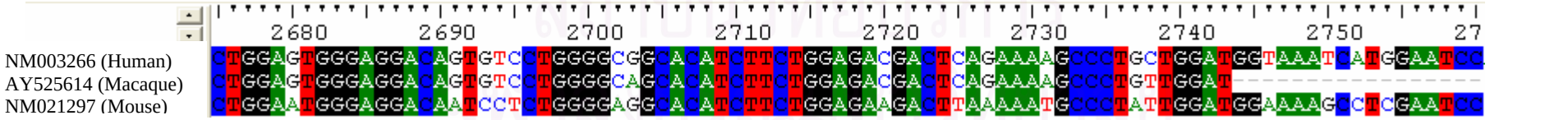


TLR4-for : **TCAGCTCTGCCTTCACTACAGA**



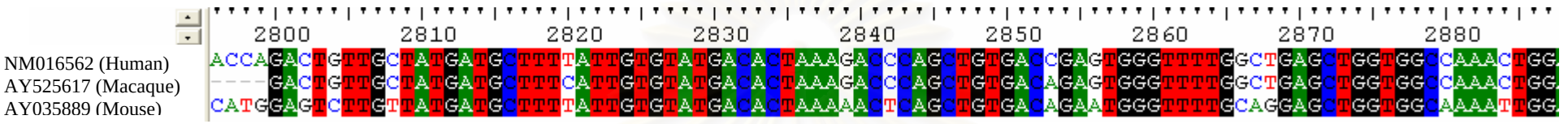


TLR4-rev : **ATG**

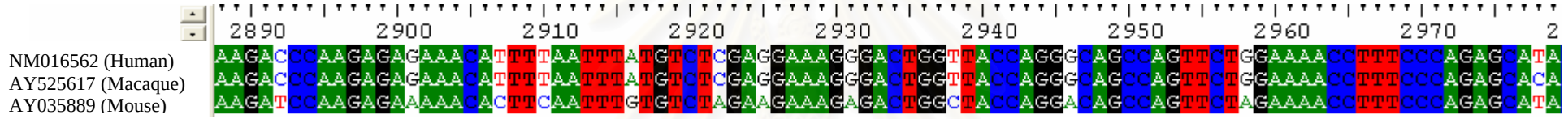


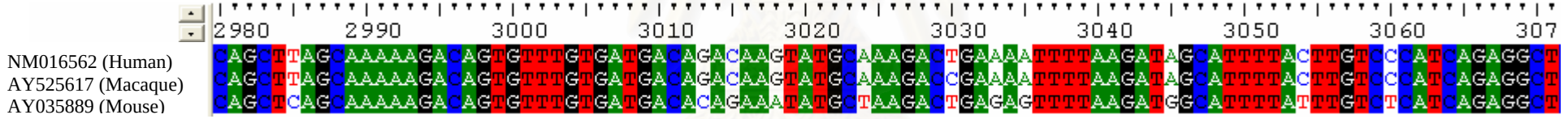
GACCTCACCCTCCTGTC

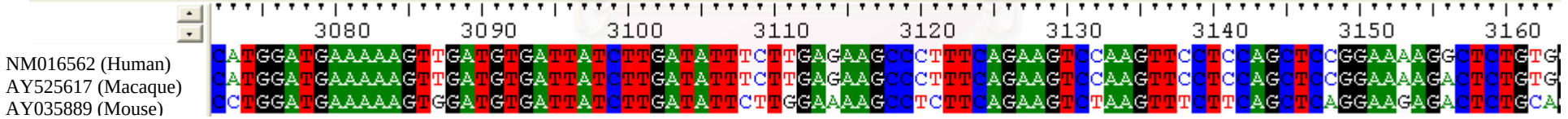
4. <u>TLR7</u> : Estimated product size = 315 bp



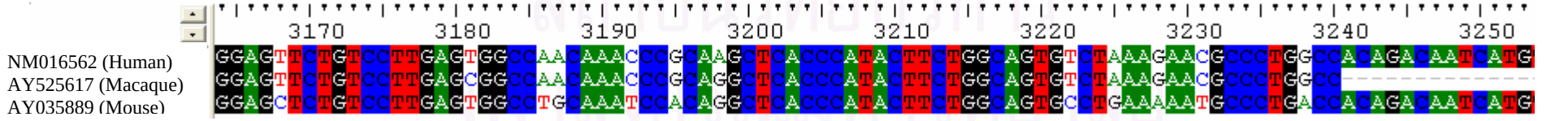
TLR7-for : **GACACTAAAGACCCAGCTGTGA**



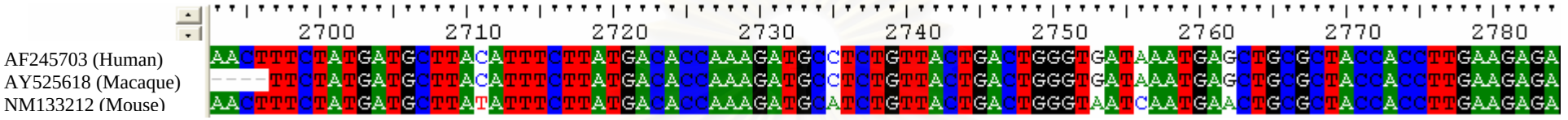




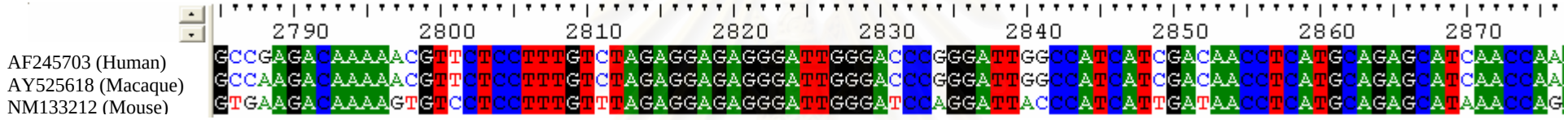
TLR7-rev : **AGTCTTCAGGTTCAAGGAGGTC**

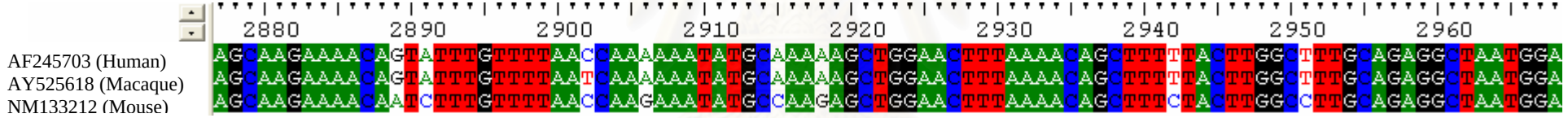


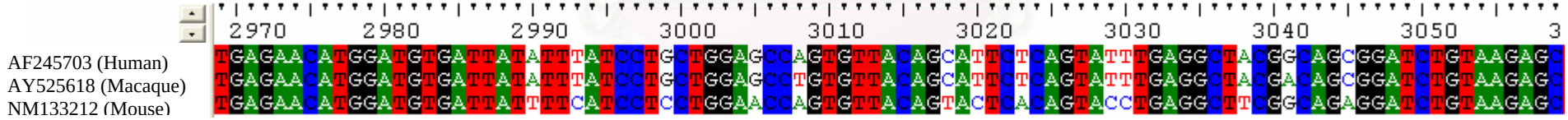
5. <u>TLR8</u> : Estimated product size = 318 bp

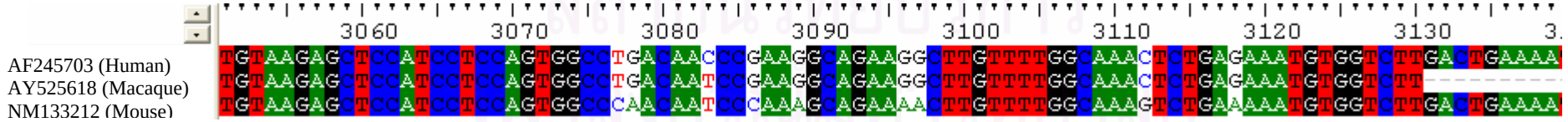


TLR8-for : **CTGCGCTACCACCTTGAAGAGA**



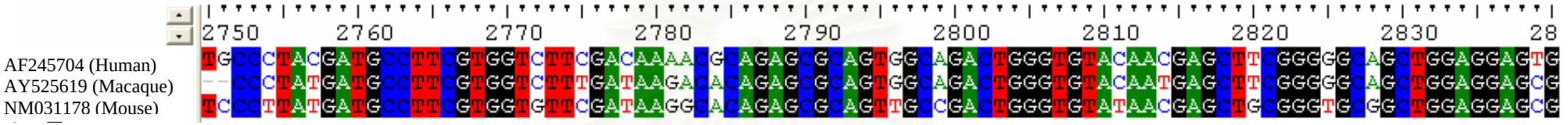


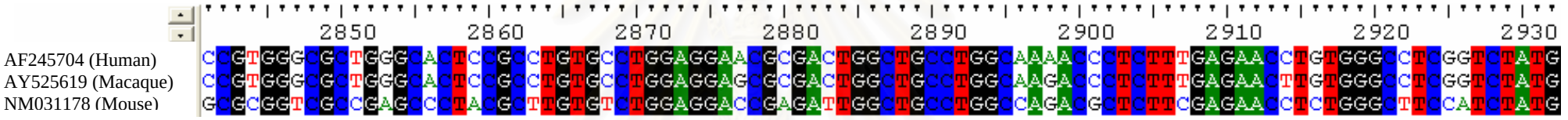


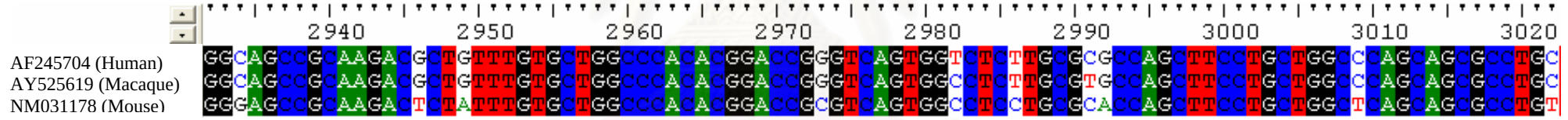


TLR8-rev : **AGGTAGGAGGTCACCGGACTG**

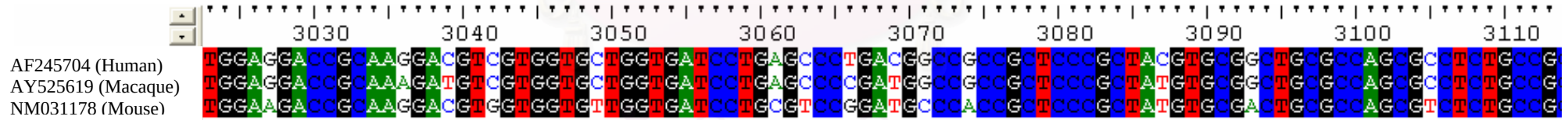
6. TLR9 : Estimated product size = 181 bp



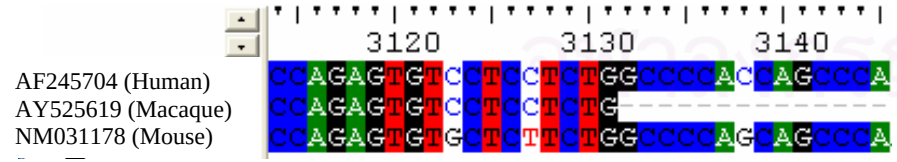




TLR9-for : **GCTGTTTGTGCTGGCCCACA**



TLR9-rev : **AGACGGC**



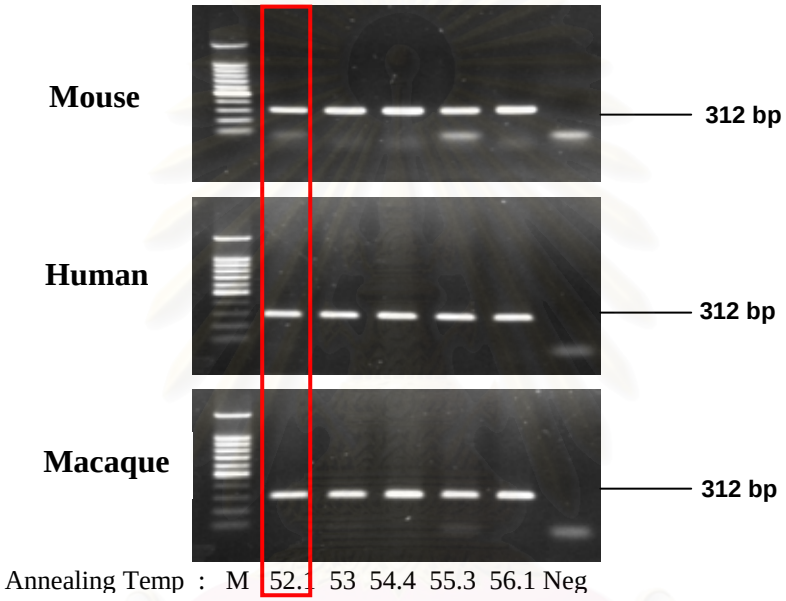
GGTCTCACAGGAG

<u>Part II</u> : PCR optimization for each of primer sets

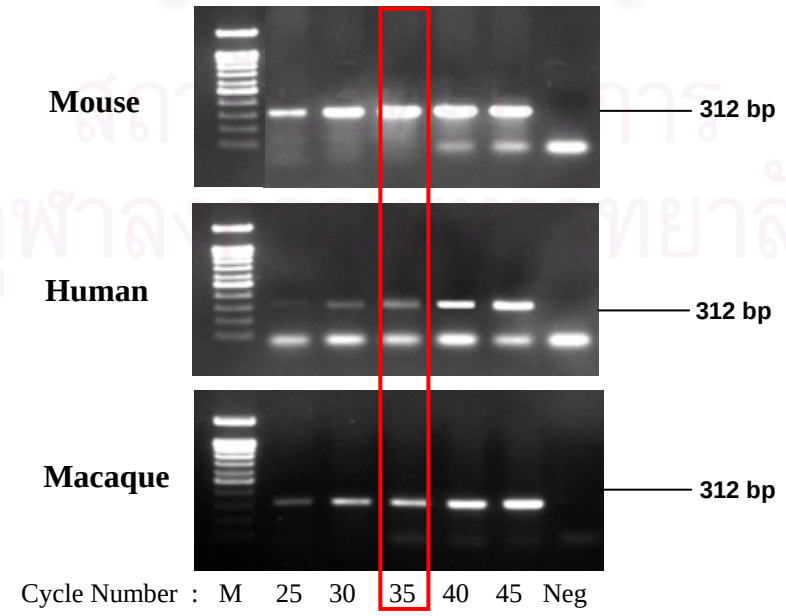
As mention in the method (Topic 3.2.2.3), the annealing temperature and cycle number were optimized for each of primer sets. Then, the sensitivity of each of primer sets was done by varying the amount of cells per reaction.

1. β-actin :

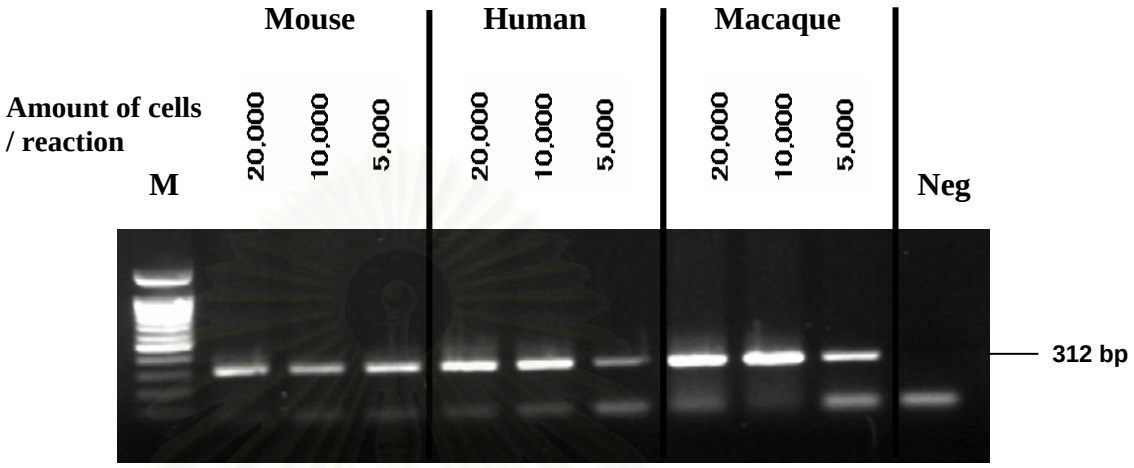
1.1 An optimal temperature is 52 ºC.



1.2 An optimal cycle number is **35** cycles.

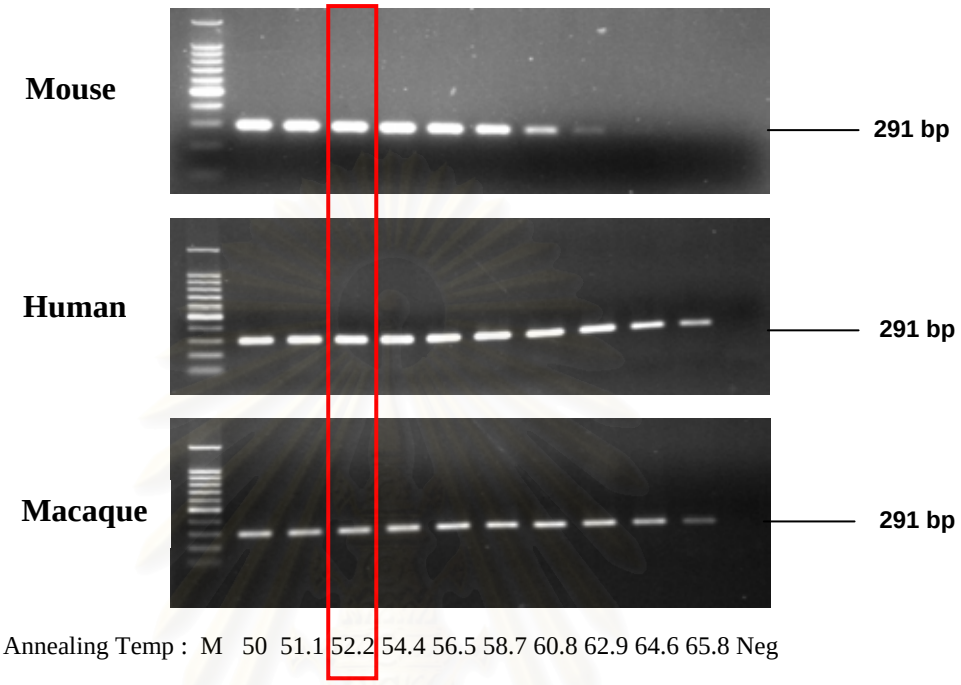


1.3 The sensitivity of β-actin primers for all three species is less than **5,000** cells per reaction.

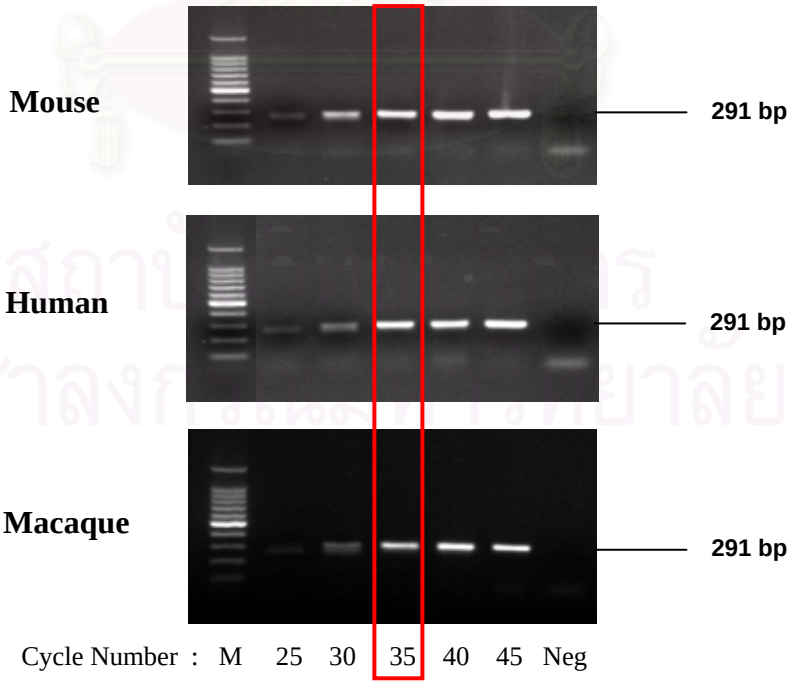


2. TLR4 :

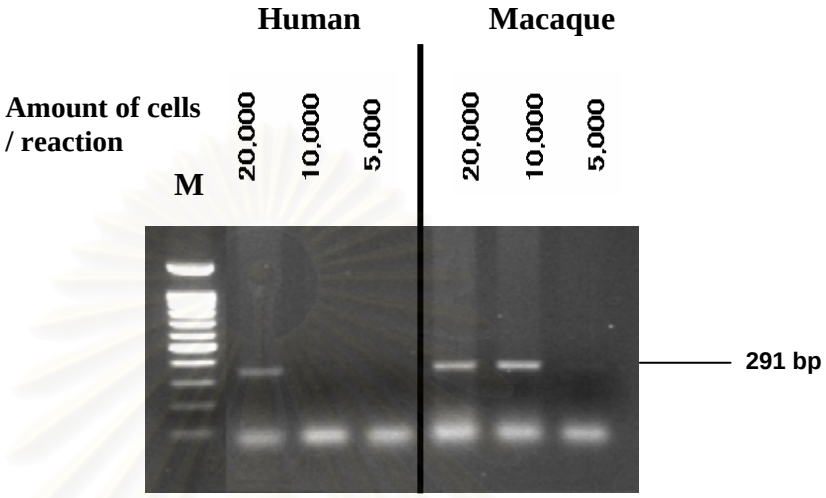
2.1 An optimal temperature is **52 ºC**.



2.2 An optimal cycle number is **35** cycles

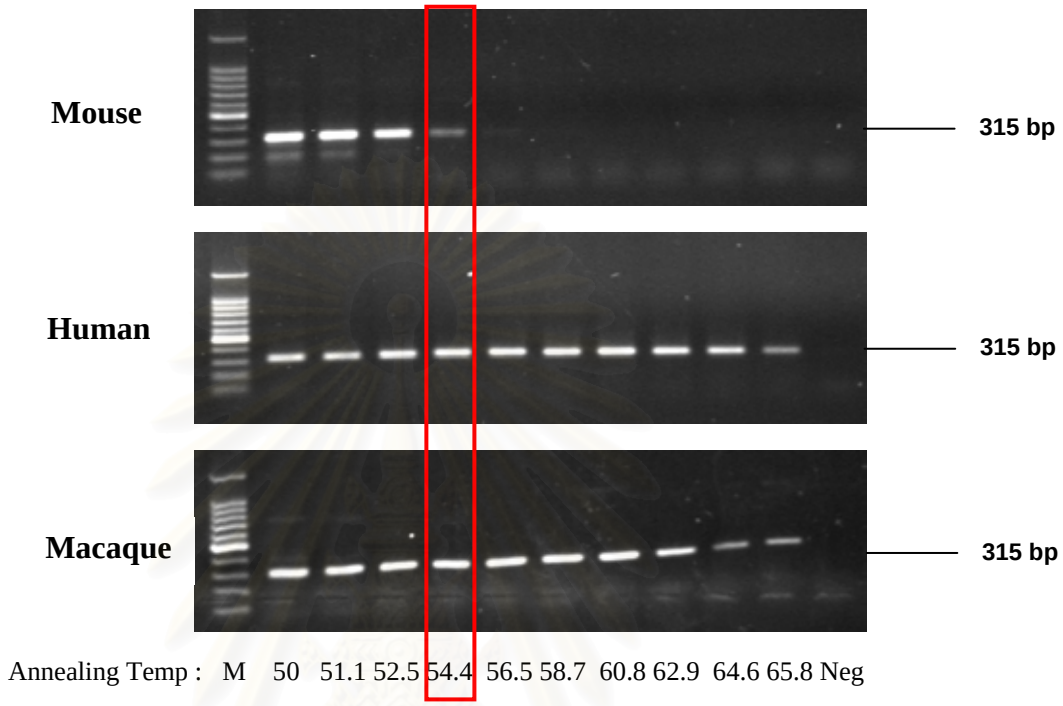


2.3 The sensitivity of TLR4 primers for human is **20,000** cells per reaction, whereas for macaque is **10,000** cells per reaction.

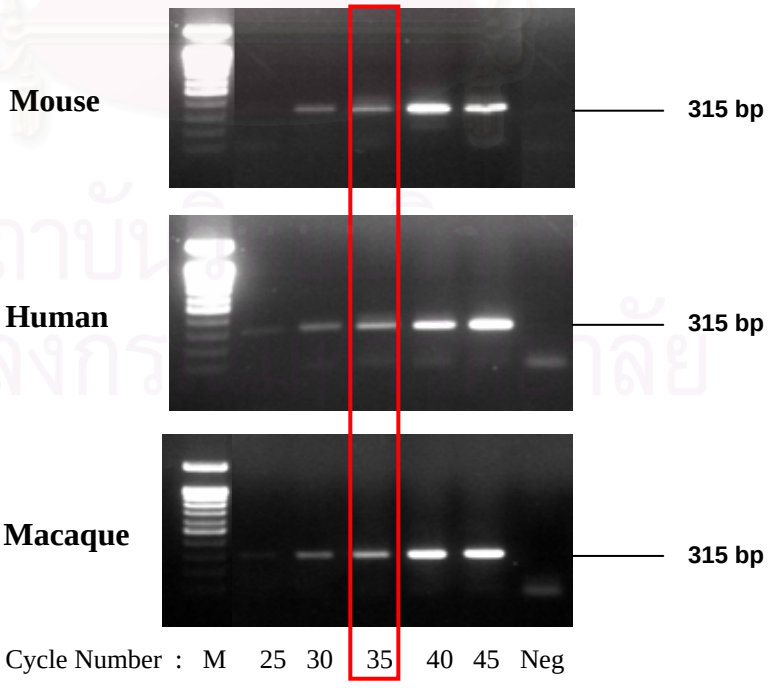


3. TLR7 :

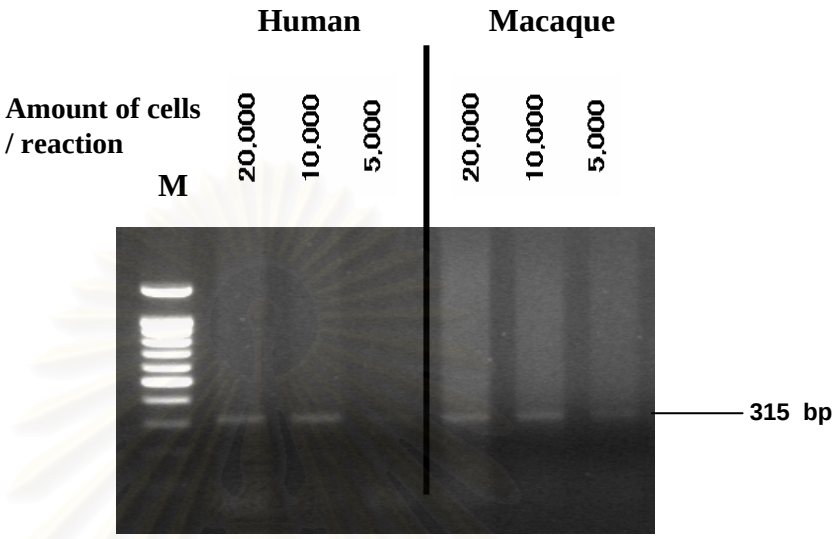
3.1 An optimal temperature is **54 ºC**.



3.2 An optimal cycle number is **35** cycles.

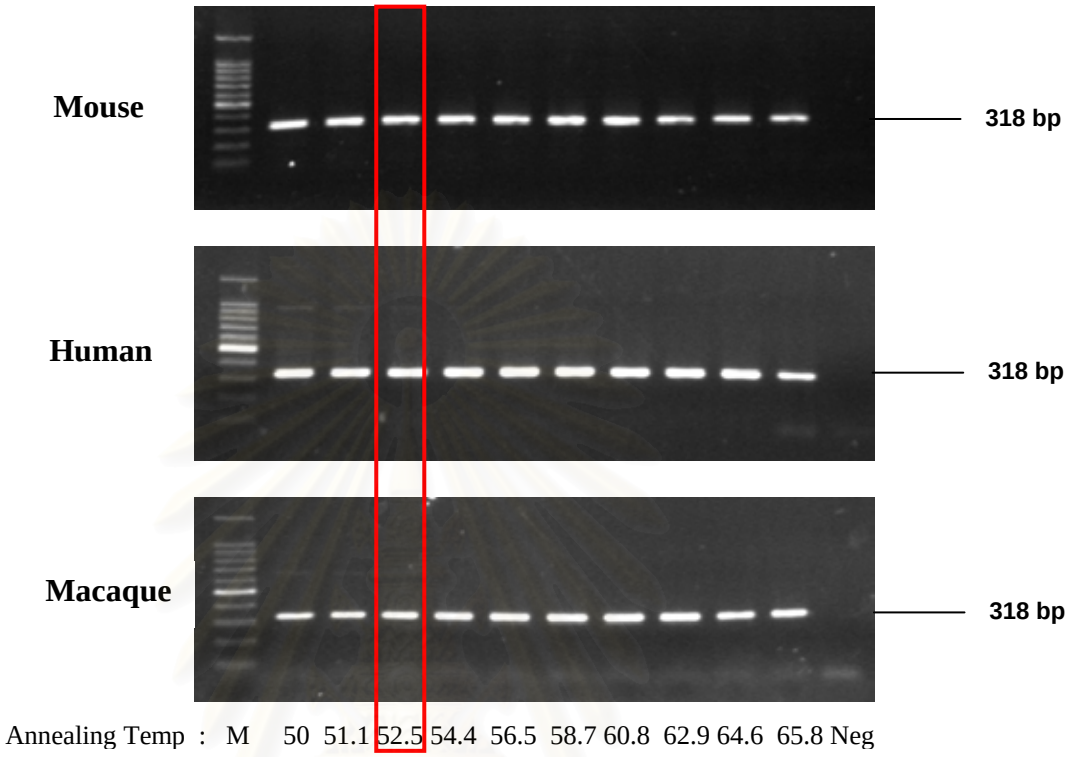


3.3 The sensitivity of TLR7 primers for human is **10,000** cells per reaction, whereas for macaque is **5,000** cells per reaction.

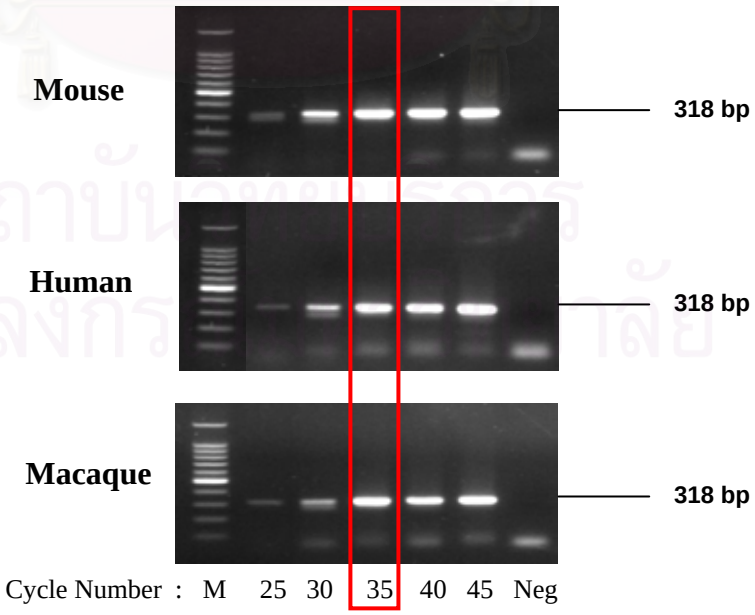


4. TLR8 :

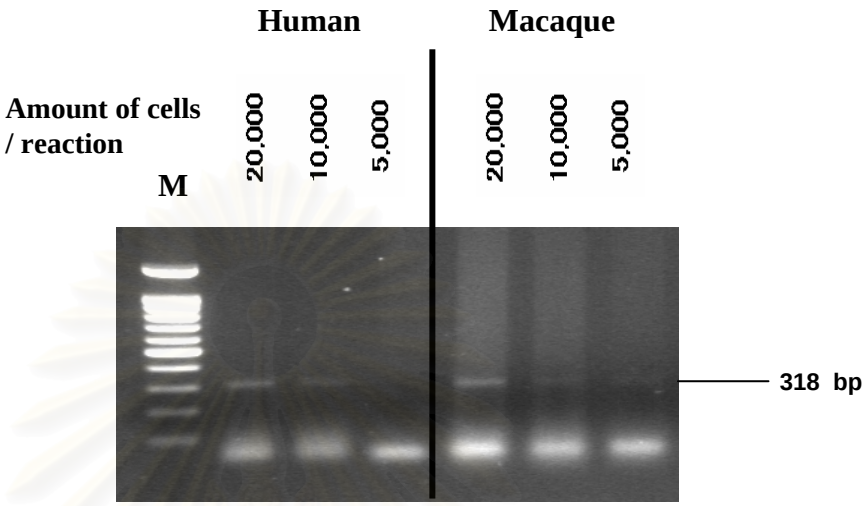
4.1 An optimal temperature is **52 ºC**.



4.2 An optimal cycle number is **35** cycles.



4.3 The sensitivity of TLR8 primers both human and macaque is **10,000** cells per reaction.



APPENDIX II

CHEMICAL AGENTS AND INSTRUMENTS

A. CHEMICAL AGENTS

100 bp DNA ladder (Promega, USA)

Agarose (GIBCO; Grand Island, N.Y. USA)

Bromphenol blue (Sigma, MO, USA)

CD1c (BDCA-1) Dendritic cell isolation kit (non-human primate) (Miltenyi Biotec, Germany)

dNTPs (Promega, USA)

Ethidium Bromide (Sigma, MO, USA)

Guanidium thiocyanate (Sigma, MO, USA)

Histopaque-1077 (Sigma, MO, USA)

Isopropanol (Sigma, MO, USA)

recombinant mouse Flt-3 ligand (R&D System, Minneapolis, MN)

recombinant human granulocyte-macrophage colony-stimulating factor (rh GM-CSF) (R&D System, Minneapolis, MN)

recombinant interleukin-4 (rh IL-4) (R&D System, Minneapolis, MN)

RNAsin (Promega, USA)

RPMI Medium 1640 (GIBCO, USA)

Tag DNA Polymerase (Promega, USA)

Trypan blue (Sigma, UK)

B. INSTRUMENTS

96- well flat plate (Costar, USA)

Agarose submarine gel apparatus

Automatic pipette (Gilson, Lyon, France)

Analytical Balance

Centrifuge

Conical tube 15, 50 mL (Falcon, USA)

Counting chamber

Cover slip

Cryotube (Sarstedt, Germany)

Electrophoresis power supply (Biorad, CA, USA)

Freezer -70^0C

Glover, sterile

Heparin tube (Becton-Dickinson, USA)

Incubator (Forma Scientific, Ohio, USA)

MACS columns (LD, MS) (Miltenyi Biotec, Germany)

MACS Separators (Miltenyi Biotec, Germany)

Microcentrifuge (Eppendorf, USA)

Microtube 250 μL, 1.5 mL

Mixer Vortex-Genic (Scientific industries, N.Y., USA)

Multichannel pipette and Pipette Tisps

PCR machine Gene Amp PCR System 2400 (Perkin Elmer)

Serological pipette 1, 2, 5, 10, 25 mL (Costar, USA)

Stereomicroscope (Olympus, Japan)

UV Trans-illuminator (ULTRA-LUM, Carson, California)

BIOGRAPHY

Miss Chutitorn Ketloy was born on July 28, 1978 in Bangkok, Thailand. She received a Bachelors degree in Medical Technology from the Faculty of Allied Health Science, Chulalongkorn University in 1998. She got her Master degree of Science in Medical Science from the Faculty of Medicine from the same University in 2001. After that, she enrolled in a graduate program for a doctoral degree in Biomedical Science, Chulalongkorn University in 2002.