# Investigate the regulatory effect of human IL-37 isoform a (IL-37a) on Toll-like receptor (TLR) response *in vitro* and *in vivo*

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A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

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November 2019

# Abstract

**Background:** Interleukin-37 is a newly discovered member of the IL-1 superfamily, consisting of 5 isoforms (IL-37a-e) due to different RNA splicing. Current studies are only focused on IL-37b isoform and demonstrate that IL-37b is an important immunosuppressive cytokine in TLR response in inflammatory diseases. However, the function and importance of other isoforms is still unknown. Here we investigate the function of IL-37 isoform a (IL-37a) which has a unique nuclear localisation sequence (NLS) and elastase cleavage sites in the protein N-terminal, while also containing the same receptor binding domain as IL-37b in the C-terminal.

**Hypothesis:** As IL-37a carries the same receptor-binding domain as IL-37b, the two isoforms may share the same receptor and signalling via similar pathway in the regulation of TLR function. However, the unique N-terminal domains may confer IL-37a additional function in nuclear translocation and gene regulation.

**Aims:**

1. To produce IL-37a and IL-37b recombinant proteins and transgenic mice. (Chapter 3).

2. To investigate the regulatory effect of IL-37a on TLR response *in vitro* and in a disease model (Chapter 4).

3. To explore the underlying molecular mechanism by which IL-37a and IL-37b differently affect TLR4 response in immune cells using transcriptomic analysis (Chapter 5).

**Results:**

My results demonstrated that:

1. The recombinant IL-37a and IL-37b monomer and dimer proteins have been successfully produced in E. *coli* and purified by chromatographs, respectively. The IL-37a protein is bioactive and able to suppress TLR-induced inflammatory

cytokine production in immune cells. The monomer IL-37 is more suppressive than the dimers. More importantly, IL-37a is more effect than IL-37b in the regulation of TLR function *in vitro*. The IL-37a transgenic mice that express human IL-37a under CAG promoter, were also generated successfully. The strain produces IL-37a in immune cells and in blood. The transgenic IL-37a is also bioactive and regulates TLR functions *in vitro* and *in vivo*.

2. IL-37a is highly induced among the 5 IL-37 isoforms in human macrophages by different TLR stimulations. IL-37a is able to suppress TLR-induced proinflammatory cytokine production *in vitro*. In addition, IL-37a is more effective than IL-37b in the suppressive effect indicating that IL-37a may be a crucial IL-37 isoform in the regulation of TLR response. TLR4/LPS signals play a critical role in salmonella infection; we found that IL-37a can suppress LPS induced inflammatory cytokine and NO production in macrophages. We further investigated its role in the regulation of TLR response in salmonella infection *in vitro* and *in vivo*. *In vitro*, IL-37 enhanced bacteria survival in infected macrophages, while reduced proinflammatory cytokine and nitric oxide (NO) production. The same effect was also found *in vivo* using recombinant IL-37 protein or transgenic mice. Using IL-37 receptor (IL-1R8) deficient mice, we demonstrated that IL-37a signals via the receptor IL-1R8 as IL-37b. Thus, IL-37 may be a double-edged sword in salmonella infection; reducing inflammation but enhancing bacteria survival.

3. Transcriptomic analysis results revealed that IL-37a has a broad regulatory effect on global gene expression induced by TLR4 signalling in macrophages, by enhancing or reducing the expression of many genes. Furthermore, IL-37a differs from IL-37b in gene regulation, while they co-regulated some genes. This confirms that IL-37a is independent of IL-37b and explains their functional similarity and difference between the two isoforms. The key signalling pathways regulated by IL-37a are also identified, including Mitogen-activated protein kinase (MAPKs), phosphatidylinositol 3-kinase (PI3K), Nuclear factor kappa-light-chain (NF-κB) and JAK-STAT pathways. Thus, IL-37a is a novel immunoregualtory cytokine in TLR response.

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## Acknowledgement

There are so many great people who have helped and supported me during this work. I would like to extend my thanks to everyone for their contribution.

Firstly, my gratitude goes to my supervisors, Dr Damo Xu, Dr Daniel Wall and Dr Charles McSharry for giving me the opportunity to undertake this research and for their excellent supervision, providing me with advice, direction and encouragement throughout. Without their support and help it would not have been possible to finish this work. I am especially appreciative of the time they devoted to redrafting and proof-reading many different versions of manuscripts and thesis chapters.

I would also like to express my appreciation to my post doctor Dr. Maria-Ioanna Christodoulou for her guidance and technical support throughout these years. I would also particularly grateful to my colleagues especially in Dr Xu's and Dr Daniel`s group and other researchers in level 4 and level 2 for the time they devoted to helping me on my research and manuscript. I would also like to express my gratitude to Dr Mohammed Talib Mohammed Almeshleb, Miss Najwa Jameel Hameed Aga, Dr Moeed Akbar, Miss Mengxi Yuan, Miss Xiang Li, Dr Yinbo Xiao, Miss Yilin Wang, Miss Luxin Yang and for the academically and personally supports throughout these years.

I am also grateful to Dr Patrizia Di Crescenzio, Dr Tomasz Guzik and Ms Diane Vaughan for providing the technical support whenever I needed it. I greatly appreciate with the help of Dr Mariola Kurowska-Stolarska and Prof Simon Milling for their permission and guidance of animal work. I would also like to say thank you to Mr Michael McKinless, Mr Colin Hughes, Mr Anthony McDermott, Colin, Margaret and all other staff in the animal research center, your patience and help were absolutely essential for me to finish the *in vivo* work throughout this thesis.

I gratefully acknowledge the funding sources that made my PhD work possible. I was funded by the China Scholarship Council for my PhD research. Finally, my special thanks go to my amazing parents, for their moral and financial support during my PhD. Their encouragement assisted me overcome moments of discouragement, and was indispensable for the completion of my PhD. For this, and for all things, I am deeply grateful to them.

## Author's Declaration

The work presented in this thesis represents original work carried out by the author, with the exception of induction, tissues collection, breeding and processing of mice samples was performed with the help of Michael Mckinless and Anthony Mcdermott (Chapter 3), and protein purification in chapter 3, which was performed under the supervision of Patrizia Di Crescenzio (University of Glasgow). The RNA-seq data from IL-37bTg mice and IL-37 transgenic RAW cells in Chapter 5 was acquired from my supervisor Damo Xu. This thesis has not been submitted in any form to any other University Where reagents, materials or technical support has been provided by others, appropriate acknowledgement has been made in the text.

Signature: …….

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## Abbreviations

ADCC – Antigen-dependent cell-mediated cytotoxicity

AKT – Protein kinase B (PKB)

AMCDC – Antibody-mediated complement-dependent cytotoxicity

ANOVA -Analysis of variance

AP – Activator protein

APC – Antigen presenting cells

APRIL – A proliferation-inducing interleukin ligand

ARG – Arginase

B cells – B lymphocytes

BAFF –B-cell-activating factor

BCR – B-cell receptor

BD- Behcet's disease

Blimp-1 –B lymphocyte induced maturation protein-1

BLNK – B-cell linker protein

BTK – Bruton's tyrosine kinase

CC – Cervical cancer

CCL – Chemokine ligand

CD – Crohn’s disease

Cdk – Cyclin-dependent kinases

cDNA – complementary deoxyribonucleic acid

CDSP – Cytotoxic DNA-sensing pathway

CFU- Colony forming units

CLP – Common lymphoid progenitor

CLRs – C-type lectin receptors

CPG – Cytosine-phosphate-guanine

CSR – Class switch recombination

CTL – Cytotoxic T lymphocyte

CXCL – CXC chemokine ligand

CXCL2 Chemokine (C-X-C motif) ligand 2

DAMPs – damage-associated molecular patterns

DCs –Dendritic Cells

DEG – Differentially expressed genes

DJ – Diversity and joining

DMEM –Dulbecco's Modified Eagle Medium

DPBS – Dulbecco's Phosphate Buffered Saline

*E. Coli – Escherichia coli*

EAE – Experimental autoimmune encephalomyelitis

EBV – Epstein-Barr virus

EDTA – Ethylenediamine tetra-acetic acid

ELISA – Enzyme linked immunosorbent assay

ERK – Extra-cellular signal-regulated kinase、

ETR – Endotoxin removal

Fab – Fragment antigen binding

FACS – Fluorescence assisted cell sorting

FBS – foetal bovine serum

Fc – fragment crystallisable

FcR – Fragment crystallisable receptor

FCS – foetal calf serum

FLIL – Full-length interleukin

FOXO- Forkhead box O

FOXP-3 – Forkhead box proteins-3

FTICR – Fourier transform ion cyclotron resonance

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GATA3 – GATA-binding factor

GC – Germinal centre

G-CSF – Granulocyte colony-stimulating factor

GD – Graves' disease

GM-CSF – Granulocyte-macrophage colony-stimulating factor

Grb-2 – Growth factor receptor-bound protein-2

GSK – Glycogen synthase kinase

GSK3 – Glycogen synthase kinase 3

GST – Glutathione S-Transferase

H2SO4 – sulphuric acid

HBsAG – Hepatitis B surface antigen

HCC – Hepatocellular carcinoma

HCl – Hydrogen chloride

HDM – house dust mite

HIV – Human immunodeficiency virus

HPV – Human papilloma virus

HRP – Horse radish peroxidase

HSC – Haematopoietic stem cell

IAV – Influenza A virus

IBD – Inflammatory bowel disease

IC – The immune complex

IFN – Interferon

IFN-β-1 – interferon-beta 1

IFN-γ – Interferon gamma

Ig – Immune globulin

IGF –Insulin growth factor

IHC – Immunohistochemistry

IKK – IκB kinase

IL – Interleukin

ILCs – Innate lymphoid cells

iNOs – inducible nitric oxide synthase

IP3 – Inositol-(1,4, 5)-P3

IPTG – Isopropyl β-D-1-thiogalactopyranoside

IRAK-1 - IL-1R associated kinase1

IRAK-4 – Interleukin receptor-associated kinase

IRES- Internal ribosome entry site

IRF – Interferon response factors

ITAM – Immunoreceptor tyrosine-based activation motif

JAK – Janus kinase

JCSG – Joint Centre for Structural Genomics

JNK – JUN N-terminal kinase

JUN – JUN proto-oncogene

KEGG – Kyoto Encyclopedia of Genes and Genomes

KO- Knockout

LAL – Limulus amebocyte lysate

LB - Luria broth

L-chain – Light chain

LPS – Lipopolysaccharide

LRR – Leucine rich repeats

LYN –Src-family kinase

M1 –Classically activated macrophages

M2 – The alternative macrophages two

MACS –Magnetic-activated cell sorting

MAL – Myeloid adaptor-like protein

MALDI – Matrix-assisted laser desorption and ionization

MAPKs – Mitogen-activated protein kinases

MBP – Maltose binding protein

MCP – Monocyte chemoattractant protein

M-CSF –Macrophage colony-stimulating factor

MEK – MAP/ERK kinase

MEKK– MAP/ERK kinase kinase

MEKKK – MAP/ERK kinase kinase kinase

MHC – Major histocompatibility complex

MHC-I – Major histocompatibility complex one

MHC-II – Major histocompatibility complex two

MIP – Macrophage inflammatory protein

MKK – Map kinase kinase

MS –Multiple sclerosis

mTOR – Mammalian target of rapamycin

MyD88 –Myeloid differentiation primary response gene (88)

NFAT–Nuclear factor of activated T cells

NF-κB –Nuclear factor kappa-light-chain

Ni – Nickle

Ni-NTA – Nickle nitrilotriacetic acid

NK– cells Natural Killer cells

NLR – Node like receptor

NLS – Nuclear localisation sequence

NMR – Nuclear magnetic resonance technique

NO – Nitric oxide

NOD –Nucleotide-binding oligomerization domain

NSCLC – Non-small cell lung cancer

OD- Optical density

OVA – Ovalbumin

Ox-LDL – Oxygenated low-density lipoprotein

PACT – PACT premier is a pH, Anion, Cation crystallization trial

PAMP – Pathogen associated molecular patterns

PBMCs – Peripheral blood monoclonal cells

PBS – Phosphate buffered saline

PCs – Plasma cells

PH – Potential hydrogen

PI- Isoelectric point

PI3– kinase Pphosphoinositide-3-kinase

PI-3K – Phosphatidylinositol-3 kinase

PiK – Phosphatidylinositol kinase

PIP-3 – Phosphatidylinositol triphosphate

PLC – Phospholipase C

PLTP – Phospholipid transfer protein

PPARr – peroxisome proliferator-activated receptors

PRRs– Pattern recognition receptors

PTKs– Protein tyrosine kinase

PTPN – Tyrosine-protein phosphatase non-receptor

qPCR – Quantitative polymerase chain reaction

RA – Rheumatoid arthritis

RANK – receptor activator of NF-kB

RANKL –receptor activator of NF-kB ligand

RAS – Reactive oxygen species

RAW – Murine macrophage cell line

RC- Renal cancer

RING – Really Interesting New Gene

RIP – Receptor interacting protein

RNA – ribonucleic acid

ROS –Reactive oxygen species

RPMI – Roswell Park Memorial Institute

RT-PCR – Reverse transcription polymerase chain reaction

*Salmonella spp.* – Salmonella species

Samd- Sterile alpha motif domain

SDS – Sodium dodecyl sulphate

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEC – Size exclusion chromatography

SHIP –SH2 domain containing inositol 5’ phosphatase

SHM – Somatic hypermutation

SHP –Src homology region 2 domain-containing phosphatase

SIg – Secretory immune globulin

SIGIRR –Single Ig IL-1-related molecule

siRNA – Small interference RNA

SLC – Surrogate light chain

SLE – Systemic lupus erythematosus

SP – Streptococcus pneumonia

SRC – Proto-oncogene tyrosine-protein kinase Src

sST– soluble suppression of tumorigenicity 2

ST2 - Suppression of tumorigenicity 2

STAT – Signal transducer and activator of transcription

SYK – Spleen tyrosine kinase

T cells – Thymus lymphocytes

TAB – TGF-beta-activated kinase 1 and MAP3K7-binding protein 2

TAK1 –Transforming growth factor beta activated kinase-1

TANK – binding kinase

TBE - Tris-borate-EDTA

TBK – kinase

TCR – T cell receptor

TD – T-cell dependent

tDC – Tolerogenic dendritic cells

Tfh- Follicular helper T cells

Tg –Transgenic

TGF – Transforming growth factor

Th cell – T helper cell

THP-1 – Human monocyte cell line

TIR – Toll interleukin resistance

TIRAP –Toll/interleukin-1 receptor adaptor protein

TKB – Tyrosine kinase-binding

TLR – Toll-like receptor

TNF – Tumour Necrosis Factor

TNFα – tumour necrosis factor-α

TRAF – Tumour necrotic factor receptor-associated factors

TRAM – TRIF related adaptor molecule

Treg – Regulatory T cells

TRIF –TIR-domain-containing adapter-inducing interferon-β

UC – Ulcerative colitis

UCMSCs – Umbilical cords-derived mesenchymal stem cells

VEGF – vascular endothelial growth factor

VEGF-C – Vascular endothelial growth factor-C

WT – Wild type

## Units

-/- Knock out gene deletion

°C Degree Celsius

µg - Microgram

µl - Microlitres

µM - Micromolar

g - Gram

kDa - Kiladaltons

M - Molar

mg - Milligram

ml - Millilitres

mM - Millimolar

ng- Nanogram

d- Days

P value (P) Probability value

rpm - revolutions per minute

SD - Standard deviation

UV - Ultraviolet

V - Volt

EU- Endotoxin unit

# Chapter 1

# Introduction

# 1. Introduction

## 1.1 The immune system

The immune system is a complex and powerful defence mechanism that has evolved to protect the body from microbial infections. Cells of the immune system recognize and induce appropriate inflammatory responses to microbial antigens which limits and resolves infection and maintains tissue homeostasis (Murphy and Weaver, 2016).

The immune system has the following main functions：

1. Immune defence; to prevent and eliminate infectious microbes, such as bacteria, viruses, fungi and parasites as well as their toxic products.

2. Immune surveillance; to recognise self-antigens and thereby to distinguish non-self-antigens and to eliminate them. An important example is the screening, recognition and elimination of apoptotic cells and of potential tumour cells caused by gene mutation or epigenetic changes associated with ageing or environment *e.g.* exposure to smoke.

3. Maintenance of immune homeostasis; this is an important function which maintains the stability of tissue homeostasis by mechanisms of immune tolerance and immune regulation (Abbas *et al.,* 2011).

The mechanisms of these functions can be summarised into two general categories:

1. Rapid responses which are encoded by the germ-line genes of host. These recognize the molecular structures of microbes and their toxins that are essential to their survival and which are different to those of the mammalian host.

2. Specific responses which are encoded by genes which might require gene-rearrangement and the synthesis and assembly of specific antigen-binding. This process requires more time and complements the rapid response described above (Murphy and Weaver, 2016).

These two categories of response represent the **innate** (non-specific) and **adaptive** (antigen-specific) immune responses. These act synergistically for optimum efficiency.

### 1.1.1 Innate immunity

Innate immunity is the body`s 'first line of defence' against pathogens. The innate immune cells can rapidly engage with microbes immediately after infection, but they do not have antigen-specific recognition and immune memory that are associated with the adaptive immune system. Innate immunity is mediated by various innate immune cells such as myeloid cells (monocytes, macrophages and dendritic cells), granulocytes (neutrophils, mast cells, eosinophils), natural killer (NK) cells and innate lymphoid cells (ILC). These immune cells lack antigen recognition receptors but express pattern recognition receptors (PRR), which can recognise pathogen associated molecule pattern (PAMP) (Murphy and Weaver, 2016).

A subset or PRR are the toll like receptors (TLR) and these play an essential role in recognizing microbial antigens. For instance, TLR4 expressed on macrophages and dendritic cells has the ability to recognize and bind to bacterial lipopolysaccharide (LPS) on gram-negative bacterial surface membrane. Binding then triggers an anti-microbial inflammatory response which is an example of the innate immune response (Akira *et al.,* 2006). Additional key components of the innate immune system include tissue barriers, innate immune cells and innate effector immune molecules (Murphy and Weaver, 2016).

#### 1.1.1.1 Tissue barriers

**Physical barrier**: the skin epithelium and mucosal membranes on digestive, respiratory and reproductive tracts comprise the physical barrier to restrict pathogen entrance.

**Chemical barriers**: the anti-microbial substances secrete from skin and mucosal tissue such as unsaturated short-chain fatty acids, lactic acid, lysozyme and antibiotic peptides (*e.g.* defensins) in saliva and in gastric, respiratory and reproductive tract secretions (Murphy and Weaver, 2016).

**Placental barrier**: Placental barrier is the defence system which prevent toxic microbes or molecules from entering the foetal circulation; this barrier includes cytotrophoblast, syncytiotrophoblast, villus mesenchyma and the foetal capillary walls.

**Haemato-encephalic barrier**: Also referred to as the blood-brain barrier, consists of several tissues such as choroid plexus capillary walls, pia mater and astrocytes together separate the circulating blood from the extracellular fluid of the brain; together restricting pathogen access.

The normal **microbiota** in the digestive and reproductive tracts function as microbiological barrier by limiting the ability of novel microbes to establish infection (Akira *et al.*, 2006).

#### 1.1.1.2 Innate immune cells

Innate immune cells include white blood cells which mainly develop from stem cell precursors in bone marrow and develop and mature in peripheral tissues where they mediate their innate immune functions. The key innate immune cells include macrophages, dendritic cells and neutrophils (Murphy and Weaver, 2016). However, certain tissue-resident macrophages develop in specific tissue sites from embryonic precursors. In contrast, innate lymphoid cells (ILCs) do not develop in bone marrow but in peripheral tissues from lymphoid progenitor T and B cells that lack antigen receptors (Abbas *et al.*, 2012).

**Macrophages**: Macrophages are generated in peripheral tissues from blood monocyte precursors which can migrate into tissues in response to locally-derived chemotactic factors. Once in the tissue the monocytes respond to signalling molecules from the local tissue stromal cells and differentiate into tissue-specific macrophages. For instance, microglial cells, osteoclasts, Kupffer cells, histiocytes and alveolar macrophages differentiate from monocytes dependent on their specific tissue environments. Macrophages play a crucial role in innate immunity. As part of the first defence in innate immunity, macrophages can engulf, digest and remove invading microbes. In addition, macrophages contribute to immune regulation, wound healing and homeostasis; for instance, macrophages can recognise and clear apoptotic cells and cell debris (Aderem and Underhill, 1999). Furthermore, macrophages can function as antigen-presenting cells (APC), which can trigger T-cell mediates adaptive immune response. Therefore, macrophages form a crucial link between innate and adaptive immunity (Yurasov *et al.,* 2005).

Tissue macrophages can differentiate into different phenotypes depending on the local conditions of activation. There are several emerging phenotypes, but the initial classification was of two subtypes which depended on the stimulation of activation. These are: M1 (classically activated) or M2 (alternatively activated) macrophages.

**M1 macrophages** differentiated in response to cytokines of a type I adaptive immune response *i.e.* IL-12, IFN-γ and microbial LPS, produced for example during inflammation and infection. This is called 'Classical activation' (Abbas *et al.,* 2012). Classically-activated M1 macrophages can produce pro-inflammation cytokines (IL-6 and TNF). They also express inducible Nitric Oxide Synthetase (iNOs) which catalyses nitric oxide (NO) production. NO is the main intracellular cytotoxin for phagocytosed microbes (Mungrue *et al.,* 2004). M1 macrophage are also tumoricidal, and dysregulation of M1 macrophages may perpetuate infection and contribute to inflammatory disease (Parisi *et al.,* 2018).

**M2 macrophages** differentiate in response to cytokines of a type 2 adaptive immune response *i.e.* IL-4 and IL-13, and this occurs during a parasite infection or an allergic response. These 'Alternative-activated' M2 macrophages produce arginase I and transforming growth factor beta (TGF-β), which are involved in wound healing and tissue repair. Aberrant activation of M2 macrophage may lead

to pathogenic tissue remodelling and fibrosis (Levings and Schrader, 1999; Alber *et al.,* 2012).

**Neutrophils**: Neutrophils are generated in the bone marrow from stem cell precursors and are the most abundant leucocyte. They are very rapid responders to infection. are very efficient at removing microbes by phagocytosis. For example, microbes that infect a wound can cause the local innate cells (*e.g.* macrophages) to release chemokines which attracts neutrophils to traverse blood vessel wall and enter the infected tissue (Godaly *et al.,* 2001). There, the neutrophils phagocytose microbes and also release enzymes and other toxic granule components to attenuate the infection. In addition, neutrophils can synthesise several pro-inflammatory cytokines and molecular mediators which drive inflammation and resolution (Abbas *et al.,* 2012).

**Dendritic cells**: Dendritic cells (DC) are a type of antigen-presenting cell (APC), mainly present in tissues which have close contact with the external environment such as the skin and the inner lining tissues of *e.g.* gut and lung. DCs can be divided into three types: (i) conventional dendritic cells, (ii) myeloid dendritic cells which act as APCs, and (iii) plasmacytoid DCs (Spears *et al.,* 2011). The main function of dendritic cells is to process and present antigen fragments to T cells to induce an adaptive response. This process includes activation and maturation of the DC by contact with presentable antigen, the DCs process these antigens in the context of the surface major histocompatibility complex (MHC-I and II) molecules and then present them to T cells to initiate an adaptive immune response. In support of this presentation, DCs provide additional co-stimulation via CD80/86 and cytokine signals (Abbas *et al.,* 2012). Furthermore, DCs can produce many anti-microbial factors to kill the intracellular pathogens such as NO and anti-microbial proteins. Therefore, dendritic cells act as a bridge between the **innate** and the **adaptive** immune systems (Plantinga *et al.,* 2010).

**Natural Killer Cells**: Natural killer (NK) cells are derived from the same common lymphoid progenitors for B and T lymphocytes. However, unlike B and T cells, they do not have antigen-binding receptors. The main function of natural killer cells is to recognize and recognise, attach and kill cancer cells or cells with intracellular microbes. Following attachment, NK cells produce cytotoxic enzymes and

molecules to destroy the membranes of these cells and induce apoptosis. Natural killer cells are important in the initial defence against viral infections and tumour cells (Abbas *et al.,* 2011).

**Eosinophils and basophils:** Eosinophils and basophils are granulocytes which target large or multicellular parasites. The granules contain preformed mediators (either eosinophil major basic proteins, or basophil type II cytokines) which are released following contact with parasites and assist eliminate the microbes. Aberrant activation of eosinophils or basophils can cause allergic-type responses and chronic disease and tissue damage (Abbas *et al.,* 2011).

**Mast cells:** Mast cells are primarily resident in mucosal membranes and connective tissues. They play a crucial role in wound healing and tissue defence system in the inflammatory response. After activation by pathogens, mast cells can produce pro-inflammatory cytokines and granules which contain chemical molecules, these together create an inflammatory cascade. They also release mediators, like histamine which can dilate blood vessels, accelerate blood flow and recruit other immune cells to the infection area (Menzies *et al.,* 2011). In general, the mast cells are mainly involved in inflammation and defence system against pathogen infection (Abbas *et al.,* 2011).

**Innate lymphoid cells:** Innate lymphoid cells (ILCs) are a group of lymphocyte, derived from the common lymphoid progenitor (CLP) and not the myeloid progenitor. Due to the lack of the recombination activating gene (RAG), ILCs do not express antigen specific receptors as in T and B cells. However, they have cell surface markers which indicate distinct innate immune cell lineages (Spits *et al.,* 2012). These cells have varying physiological functions; some works as helper T cells to assist the activity of other cells by cytokine expression; others can express MHC class II and serve as APCs. Based on their cytokine profiles and transcription factors, ILCs can be further classified into three subgroups, (Moro *et al.,* 2010).

**ILC1s** are the major population of ILC and have anti-tumorigenic potential. Following IL-12 and IL-18 stimulation, ILC1 express the transcription factor T-bet and produce Th1 cytokines, for instance, Interferon gamma (IFN-γ) and TNF. ILC1s are analogous to Th1 cells and share the common transcription factor T-bet. They are crucial in protection against intracellular infection and have anti-tumorigenic

potential. However, ILC1 over-activation may contribute to inflammatory disorders e.g. inflammatory bowel disease (Gordon *et al.*, 2012).

**ILC2s** are derived from precursors in bone marrow that have high expression of GATA3 (Walker *et al.*, 2013). They play a key role secreting type 2 cytokines in response to helminth infection. In response to IL-25 and IL-33 activation, ILC2s produce Th2 cytokines, for example, IL-5, IL-9 and IL-13. Therefore, ILC2s are similar to Th2 cell function and respond to parasite infection. ILC2 dysregulation leads to overexpression of type II cytokines, aberrant inflammation and remodelling (Spits *et al.*, 2013).

**ILC3s** are the innate counterpart of Th17 cells and share the common transcription factor RORγt. Following IL-23 and IL-1β activation, they produce the Th17 cytokines IL-17A and IL-22. Unlike NK cells, ILC3s do not have cytotoxic function and do not express IFN-γ or TNF, but dependent on transcription factor RORγt, ILC3 are crucial in defence against extracellular pathogens. Aberrant activation of ILC3 may cause autoimmune disorders e.g. psoriasis (Spits *et al.*, 2013).

### 1.1.2 Adaptive immunity

The innate immune system plays a key role of rapid detection and elimination of pathogens. However, this mechanism is not antigen-specific. In support of the innate immune system, mammals have evolved an adaptive immune system which provides a more flexible and finely-tuned repertoire recognising self- and nonself-antigens (Murphy and Weaver, 2016). The adaptive immune response is mediated by T and B lymphocytes which express antigen receptors. B cells mediate antibody production (called humoral immunity), and T cells produce regulatory and inflammatory cytokines (called cell-mediated immunity) (Sattler *et al.*, 2013). T and B lymphocytes cells differentiate in primary lymphoid tissues (bone marrow and thymus) and mature in secondary lymphoid tissues (spleen and lymph node) which promote antigen-specific immune effector pathways, generation of immune memory, and regulation of host immune homeostasis (Murphy and Weaver, 2016).

The adaptive immune response can be divided into four phases:

1. **Initiation (priming) phase**: In this phase, T lymphocytes and B lymphocytes recognise antigens via specific antigen receptors called the T-cell receptor (TCR) and B-cell receptor (BCR) respectively.

2. **Activation and differentiation phase**: In this phase, lymphocytes receive antigen co-stimulation and cytokine signals from antigen-presenting cells (APCs). These signals initiate the activation, proliferation and differentiation of antigen specific T and B lymphocytes into their respective effector cells.

3. **Effector phase**: T and B effector cells produce effector molecules (e.g. cytokines) to eliminate microbes.

4. **Recovery phase**: in this phase following resolution of infection, the stimulation of most effector cells is reduced. Without this stimulation most effector cells become apoptotic and are pagocytosed by macrophages. Some effector cells are retained in lymph node niches and develop into memory cells.

In summary, the adaptive immune response comprises a cellular immune response of T and B lymphocytes activation, and a humoral immune response with antibody production (Murphy and Weaver, 2016).

#### 1.1.2.1 T cell mediated immunity

T lymphocytes originate from a bone-marrow common lymphoid progenitor and mature in the thymus and express the T cell receptor (TCR). After receiving the antigen detection signal from APCs, the mature antigen-naive T cells differentiate into effector cells in the peripheral lymph organs. On their surface membrane, T cells express either CD8 or CD4 glycoproteins. The CD8 positive T cells are called CD8+ cytotoxic T cells, and the CD4 positive T cells are called CD4+ helper T cells. These effector T cells can mediate the adaptive cellular immune response via antigen-dependent ligation of their surface receptors and secretion of effector molecules; mainly cytokines and chemokines, which play a key role in defence against pathogens, particularly intracellular microbial infections (Delves *et al.*, 2017).

The cellular immune response is initiated by activation of naive T cells which receive T cell receptor (TCR) signals by interacting with antigen-presenting cells (APCs). In order to induce optimal activation and differentiation of naive T cells into effector T cells, the naïve T cells need three signals from APCs. The first signal involves T cell receptor recognition of processed peptide antigens presented by APCs via their MHC molecules. The second signal results from the interaction of ligand/receptor pairs of co-stimulatory molecules on both APC and T cells. These include CD80 on APC binding to CD28 on T cells and forming a bridge between these cells. These two signals are sufficient to activate and proliferate T cells, the differentiation from native T cells into effector T cells requires a third signal. This is provided by APC-derived cytokines. With these three activation signals, naive CD8+ T cells can differentiate into effectors CD8+ T cells which have effector function. These include cytotoxic clearance of virally-infected cells and tumour cells. These three activation signals can differentiate native CD4+ T cells into several T helper cell subsets, dependent on the regulatory cytokine signals they receive and the effector cytokines they produce in response.

#### 1.1.2.2 T cell subsets

**CD4+ T cells**

The precursor CD4+ (Th0) lymphocytes can differentiate into several distinct subsets dependent on their specific cytokine activation environment. The subsets are morphologically indistinguishable but are distinguished by their profile of functional cytokine production. CD4+ T helper (Th) cells are subdivided into different subsets including Th1, Th2, Th9, Th17, Th22, Treg (regulatory T cells) and Tfh (follicular helper T cells). These subsets are characterized by their distinct profiles of cytokine production. These Th cells are crucial in the immune and effector response functions (Raphael *et al.*, 2015).

Th1 cells, when activated by IL-12 and IFN-γ, release IFN-γ and TNF which are crucial in the response to infections with intracellular pathogens, and when aberrant may cause inflammatory disease (O'Shea and Paul, 2010).

Th2 cells differentiate in response to IL-4 and produce cytokines IL-4, IL-5 and IL-13. This response is associated with protection against parasite infection. When

this response is aberrant Th2 activation may cause asthma and allergy (Golubovskaya and Wu, 2016).

Th17 cells are characterised by production of IL-17 and IL-22 when activated by IL-1 and IL-6. IL-17 is an important cytokine in host defence against extracellular bacterial and fungal infection. Dysregulation of Th17 response is associated with inflammatory and autoimmune diseases (Weaver *et al.*, 2013).

Treg cells are a crucial Th cell subset. They are specialized T cells which act to suppress immune response and maintain immune balance and immune tolerance (O'Shea and Paul, 2010). Recent study confirmed that several signals (ie. TGFβ and IL-2) are important in differentiating Tregs from naïve CD4+ cells and are essential in maintaining Treg homeostasis (Chen, 2011).

Th9 cells are a recently described effector T cells subsets. They differentiate from naive T cells by TGFβ and IL-4 signalling and can express cytokine IL-9 (Kaplan, 2013). Tfh (Follicular B helper T cells) are antigen-experienced CD4+ T cells which exist in B cell follicles, mainly identified by the expression of IL-21 and the presence of B cell follicle homing receptor CXCR5. These cells are important in affinity maturation, germinal centre formation, and the development of memory B cells (Crotty, 2014).

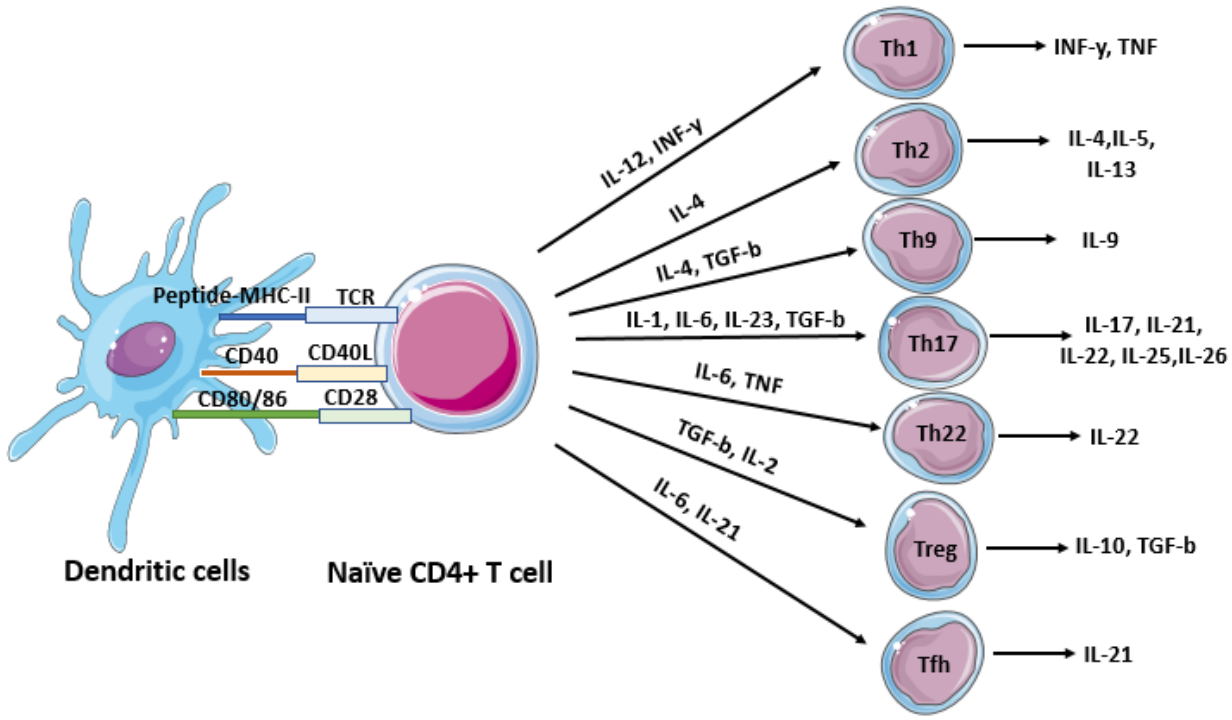



**Figure 1.1 CD4+ T cell subsets**

(Modified from Golubovskaya V, Wu L. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. *Cancers*. 2016 Mar;8(3):36.)

**CD8+ T cells**

Naive CD8+ T cells can differentiate into subsets with distinct function. These include effector T cells, effector memory T cells, central memory T cells and stem cell memory T cells. These functions are associated with different CD markers during the differentiation process.

CD8+ T effector cells are directly cytotoxic to virally infected cells and tumour cells. Their cytotoxic function involves increased synthesis of perforins and granzymes. They insert the perforins into the cell membrane of target cells and activate their caspase enzymes which cause DNA fragmentation and cell apoptosis. The cytotoxic T cells have Fas ligand on their membranes which can bind to the Fas molecules on the target cell surface and further activate apoptosis.

CD8+ effector T cells have a short half-life, however some of them differentiate into long-lived memory cells and can rapidly expand upon cognate antigen re-exposure. These memory CD8+ T cells are essential in the defence against pathogen reinfection.

### 1.1.2.3 B lymphocyte-mediated humoral immunity

In adaptive immunity, the humoral immunity driven by B cells and their antibody and cytokine products play a key protective role in the defence against extracellular pathogens. B cells produce antibodies which can neutralise toxins, prevent pathogens adhering to tissue surfaces, activate complement, opsonise bacteria for phagocytosis, and sensitise tumour or infected cells for later removal by antibody-dependent cellular cytotoxicity by killer cells.

B cells differentiate in the bone marrow from multipotent hematopoietic stem cells. B cells function primarily in secondary lymphoid structures e.g. lymph nodes where they are stimulated by cytokines released from T helper cells following APC-antigen presentation. Following B cell activation, they differentiate into plasma cells which produce antibodies. B cell activation can be either T cell dependent or independent. In the T cell dependent response, the activation of B cells requires signals from T cells in two steps:

1. Antigen recognition and activation: Initially, B cells recognise antigen by their B cell receptors (BCRs), followed by interaction with cognate T cells in the lymphoid follicles. After antigen recognition, co-stimulation of surface CD40 on B cells by binding with surface CD40L on T cells drives B cell activation.

2. B cell differentiation: Thereafter, with the assistance of T cell cytokines e.g. IL-4, activated B cells can differentiate into plasma cells with clonal expansion of those recognising antigen. This is a rapid process which can produce a substantial number of B effector cells in a short time. These B effector cells are mainly plasma cells with short half-life, which produce antibodies, express immunomodulatory cytokines and work as antigen-specific APCs in the peripheral circulation. In addition, some B cells can differentiate into memory B cells which do not produce antibodies but have immune memory functions. These reside in the germinal centre niches and can be rapidly activated to produce large amounts of antibody, and this is recognised as a secondary antibody response (Liu *et al.,* 1991; Hardy and Hayakawa, 2001).

#### 1.1.2.4 B cell development

B cells are differentiating along the lymphoid lineage and are derived from common lymphoid progenitors. B cells develop in the yolk sac and foetal liver before birth and are restricted to the bone marrow after birth in humans. In the bone marrow, signals from stromal cells activate the differentiation of the common lymphoid progenitors into pro-B cells. These signals are derived from direct contact with stromal cells and from locally secreted differentiated factors e.g. IL-7 which is an early stage B-lineage growth factor (Namen *et al.,*1988). These pro-B cells further develop into pre-B cells with gene segments encoding the κ and λ chains rearrangement, then the IgM isotype will be product on their surface after combination with μ H-chain and become immature B cells. These three stages (pro-B, pre-B and mature B cell stage) encompass the development of B cells.

1. Pro-B cell stage: At the pro-B cell stage, the gene for the heavy chain (H-chain) of the B-cell receptor starts to rearrange, then the genes for the short DH (diversity) and JH (joining) regions are recombined dependent on the recombination-activating gene enzymes (RAG1 and 2). Successful gene

rearrangements terminate the production of intact μ H-chain whereas the abortive H-chain production leads to the elimination of pro-B cells. These associate with the production of Igα and Igß heterodimers and the development of pre-B cells.

2. Pre-B cell stage: At the pre-B cell stage, the newly rearranged heavy chains are reacted with germline-encoded λ5 and Vpre-B chains (surrogate light chains). After the successful reaction between the heavy chain and the surrogate light chains, pre-B cells can express pre-B cell receptors on their cell surface membranes associated with Igα and Igß proteins which become the functional pre-B cell receptor which can transduce signals to trigger further pre-B cell proliferation (Schlissel and Baltimore, 1989; Meffre and Wardemann, 2008). Stimulation of the pre-B cell receptors assists the development of pre-B cells into immature B cells, followed by rearrangement of the Ig light chain sites, leading to random antigen specificity and increasing B cell repertoire diversity (Schlissel and Baltimore, 1989; Nishimoto *et al.*, 1991; Lassoued, 1993). Therefore, the recombination and BCR expression on immature B cell surfaces enable them to recognize specific antigens which is the main function of B cells.

3. Mature B stage: The heterogeneous immature B cell populations are refined by negative selection to remove auto-reactive B cell clones and the risk of autoimmune disease. In this process, the immature B cells are exposed to self-antigen on the neonatal thymus epithelium and in that environment the auto-reactive B cells can be eliminated by receptor editing or clonal deletion. This way, the B cells which do not recognize self-antigen will survive to become mature B cells. These then migrate into the peripheral tissues. These mature B cells are quiescent non-dividing cells with a short half-life after which most will become apoptotic unless they come into contact with antigen. Following antigen contact in lymph nodes or spleen, which provides the environment with APCs and T-helper cells and cytokines, the B cells survive, become activated and differentiated into immune B cell subsets (effector, plasma and memory B cells) (Liu *et al.*, 1991).

#### 1.1.2.5 B cell subsets

Mature B cells can be categorised into two major subsets: B1 cells and B2 cells. B1 cells (CD19+CD5+) are found mainly in the peritoneal and mucosal cavities. They comprise ~5% of the total B cell population and function primarily in innate immunity because they do not possess RAG genes and produce abundant natural IgM and low levels of IgD. They express several surface markers, such as CD11, CD54. B1 cells have low expression level of BCR on their surface and the natural IgM has typical low affinity and broad specificity for microbial antigens. These have a powerful protective function in the innate immune response to infectious disease such as streptococcus infection.

B2 cells (CD19+CD5-) are the major functional B cells. They produce high affinity antigen-specific antibodies against antigens in secondary lymphoid organs (lymph nodes and spleen). They express high level of antibodies (mainly IgG and IgE), which can execute somatic hyper-mutation (SHM) and class switch recombination (CSR) for affinity maturation. B2 cells have three main functions: cytokines secretion (e.g. IL-4 and IL-6), serving as APC and producing antibody (e.g. IgM, IgG and IgA).

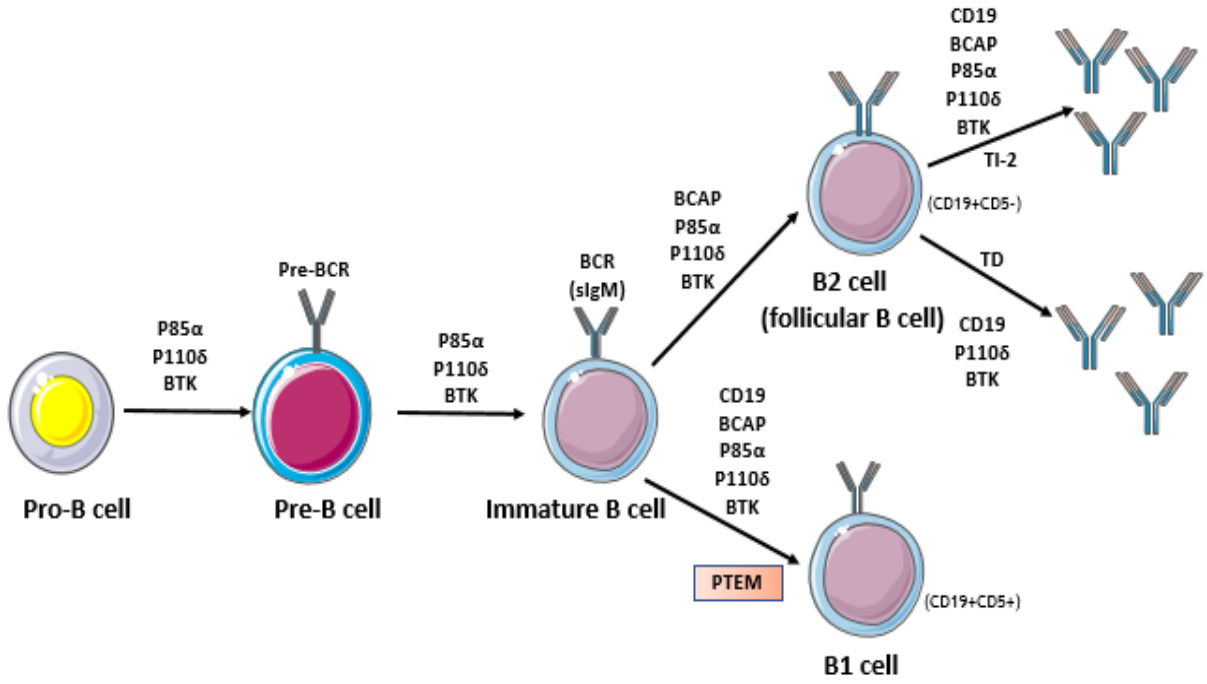



**Figure 1.2 B cell development and B cell subsets**

(Modified from Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol*. 2003; 3: 317-30)

## 1.2 Cytokines

Cytokines are small glycoproteins (8-40kDa) which have regulatory function in the immune system. They are produced by various nucleated cells either constitutively or more often following activation, e.g. in response to microbial infection (Thomson and Lotze, 2003). Currently, more than 200 molecules are identified as cytokines, including 38 interleukins. Cytokines are key mediators that regulate immune and inflammatory responses. Some can amplify and coordinate appropriate response patterns of immunity to prevent the host against threats from different pathogens and virulence factors (Holdsworth and Gan, 2015). Cytokines are molecules that mediate communication between cells. They are released by cells in response to infection or tissue damage and can bind to cytokine-specific receptors (or co-receptors) on responder cells which then perform pleiotropic functions depending on the stimulus and tissue microenvironments. Cytokines seldom work function in isolate, instead they work as an evolving cytokine network that influence the production of other cytokines to regulate the response to, and then resolution of inflammation (Marshall, 1992). However, when the host is exposed to chronic infection e.g. tuberculosis, or chronic stimulation e.g. autoimmunity, cytokine production may be overexpressed and lead to tissue damage.

Cytokines can be divided into different subtypes based on their cellular source, functions and structure.

According to their cellular sources, cytokines can be classified into three groups:

1. Monokines: cytokines produced by monocytes or macrophages

2. Lymphokines: cytokines produced by lymphocytes

3. Interleukins (ILs): cytokines mainly produced by leukocytes

According to their function, cytokines can be classified into four groups:

1. Pro-inflammatory cytokines: expressed during inflammation and in response to acute inflammation, e.g. IL-1 and IL-6.

2. Anti-inflammatory cytokines: inhibiting inflammation and supporting tissue healing, e.g. IL-10.

3. Growth factors: key signals for cell growth and survival, e.g. Granulocyte-colony stimulating factor (G-CSF) and Granulocyte-macrophage colony stimulating factor (GM-CSF).

4. Chemokines: participating in basal leukocyte migration and attracting immune cells into tissue sites of inflammation by chemotaxis, e.g. C-X-C motif chemokine 10 (CXCL10).

According to their structure, they can be classified into 9 cytokine families:

1. The haematopoietin family (IL-2, IL-4, IL-5,IL-7, IL-13, IL-15 and IL-21).

2. The Interferon family (IFNα, ß and γ).

3. The tumour necrosis factor (TNF) family (TNFα, LTα and ß, RANKL, APRIL).

4. The IL-1 family (IL-1α, IL-1ß, IL-1Ra, IL-18, IL-33, IL-36α, IL-36ß, IL-36γ,

   IL-36Ra, IL-37, IL-38).

5. The IL-17 family (IL-17 and IL-25).

6. The IL-10 family (IL-10 and IL-22).

7. The IL-12 family (IL-12, IL-23, IL-35, IL-27).

8. The TGF family (TGF-α and ß).

9. The chemokine family (CCL1- 28, CXCL1-17, XCL1 and 2, CX3CL1).

| Functional class | Primary property | Other effects | Examples |
|---|---|---|---|
| Lymphocyte growth factors | clonal expansion | Th1/Th2/Th17 polarization | IL-2, IL-4, IL-7, IL-15 and IL-17 |
| Th1 cytokines | ↑ Th1 responses | clonal expansion of CTL | IL-2, IL-12, IL-18. IL-27 and IFN-γ |
| Th2 cytokines | ↑Th2 responses | ↑ antibody production | IL-4, IL-5, IL-18, IL-25 and IL-33 |
| Th17 cytokines | ↑Th17 responses, IFN-γ | autoimmune responses | IL-17, IL-23 and IFN-γ |
| Pro-inflammatory cytokines | ↑inflammatory mediators | ↑ innate immune responses | IL-1α, IL-1β, IL-12, IL-18, IL-23, MIF, IL-32, IL-33, IL-36α/β/γ, CD40L and TNFα |
| Anti-inflammatory cytokines | ↓ inflammatory genes | ↓ cytokine-mediated lethality, ↓autoimmune diseas | IL-10, IL-13, IL-22, IL-1Ra, TGF-β, IL-36Ra, IL-37, IL-38 |
| Adipokines | pro-inflammatory | pro-atherogenic, anti-inflammatory | IL-1α, TNF-α, IL-6, leptin, adiponectin, resistin |
| gp130 signalling cytokines | growth factors | B cell activation, acute phase | IL-6, CNTF[c], IL-11, LIF[c], CT-1[c] |
| Nerve growth factors | ↑ nerve/Schwann cells | B cell activation | BNDF[c], NGF[c] |
| Osteoclast activating cytokines | bone resorption | immune stimulation | RANKL[c] |
| Colony-stimulating factors | haematopoiesis | pro- and anti-inflammatory | IL-3, IL-7, G-CSF, GM-CSF, M-CSF |
| Angiogenic cytokines | neovascularization | pro-metastatic | VEGF[c], IL-1, IL-6, IL-8 |
| Mesenchymal growth factors | fibrosis | pro-metastatic | FGF, HGF, TGF-β, BMP[c] |
| Type II IFN | macrophage activation | ↑ MHC class II | IFN-γ |
| Type I IFN | anti-viral, ↑ MHC class I | anti-inflammatory, anti-angiogenic | IFN-α, IFN-β |
| Chemokines[b] | ↑ cellular emigration | ↑ cell activation | IL-8, MCP-1, MIP-1α, others |

**Table 1.1 Functional classification of cytokines and receptors**

[a] does not include soluble cytokine receptors such as sTNFRp55, sTNFRp75, sIL-1R type II, IL-18 binding protein, osteoprotegerin.

[b] the chemokine family includes CC and CXC chemokines with over 30 members.

[c] BMP, bone morphogenic protein; BNDF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; LIF, leukaemia inhibitory factor; NGF, nerve growth factor; RANKL, receptor activator of NF-κB ligand; VEGF, vascular endothelial growth factor.

(Adapted from Dinarello, C. A. 2007. Historical insights into cytokines. *Eur J Immunol*, 37 Suppl 1, S34-45).

### 1.2.1 IL-1 family and receptors

The IL-1 superfamily is a group of cytokines that have similar protein structures and have essential roles in regulating the innate immune response (Figure 1.3). Currently there are 11 molecules in this superfamily, and the majority are pro-inflammatory cytokines (Nold *et al.,* 2010). All IL-1 superfamily members have the IL-1-like domain structure (Garlanda *et al.*, 2013). However, due to the lack of a signal peptide, these cytokines cannot be secreted via the endoplasmic reticulum and Golgi apparatus, instead, they are released by a less conventional protein secretory pathway, via exosomes, secretory lysosomes or exocytosis vesicles (Carruth *et al.,* 1991).

Several cytokines in the IL-1 family have dual functions; following transcription they can function as a nuclear transcription factor e.g. cytokine IL-33; or following secretion they can function as cytokines e.g. IL-33 and IL-37 (Boraschi *et al.,* 2011; Roussel *et al.,* 2013). The IL-1 family members can be subclassified into three groups, according to the size of the N-terminal pro-pieces that remains after cleavage of the complete ligands. These are the IL-1 subfamily, the IL-18 subfamily and the IL-36 subfamily (Garlanda *et al.,* 2013, van de Veerdonk and Netea, 2013).

The IL-1 subfamily contains three group of cytokines: IL-1$\alpha$, IL-1$\beta$, and IL-33 have the longest pro-pieces containing around 270 amino acids. In the IL-1 subfamily, IL-1$\alpha$ and IL-1$\beta$ transmit signals via their common receptor IL-1R, which is a heterodimer of the IL-1 receptor accessory protein (IL-1RacP) and the IL-1R1. IL-33 signalling is mediated via IL-1LR4 (ST2) and IL-1RAcP (Schmitz *et al.,* 2015).

The IL-18 subfamily includes IL-18 and IL-37, which have long pro-pieces of approximately 190 amino acids length. The cytokine receptor of the IL-18 subfamily consists of IL-18R$\alpha$ and its accessory chain IL-18R$\beta$ which together mediate pro-inflammatory signalling and TIR8 (SIGIRR) required after IL-37- IL-18R$\alpha$ binding and regulate the inflammation of the host, which will be described later in detail.

The IL-36 subfamily, IL-36$\alpha$, IL-36$\beta$, IL-36$\gamma$, and IL-38 have smaller pro-piece lengths of approximately 150 amino acids. All the members of IL-36 subfamily bind

to the IL-36R, except IL-38. The IL-36/IL-36R binding also recruits co-receptor IL-1RAcP for signalling (Dinarello, 2009). IL-38, also known as IL-1HY2, which shares similar structure as IL-1 receptor antagonist (IL-1Ra) and IL-36Ra. IL-38 is a candidate in inflammation, and functions mainly to inhibit IL-17 and IL-22 expression in Th1 responses. Moreover, IL-38 also suppress the production of IL-8, to inhibit inflammatory responses. The putative receptors of IL-38 are IL-36R and IL-1RAPL1 (Figure 1.3).

In summary, the IL-1 family is an important component of the first-line immune defence system against pathogenic micro-organisms and tissue damage (Veerdonk and Netea, 2013).

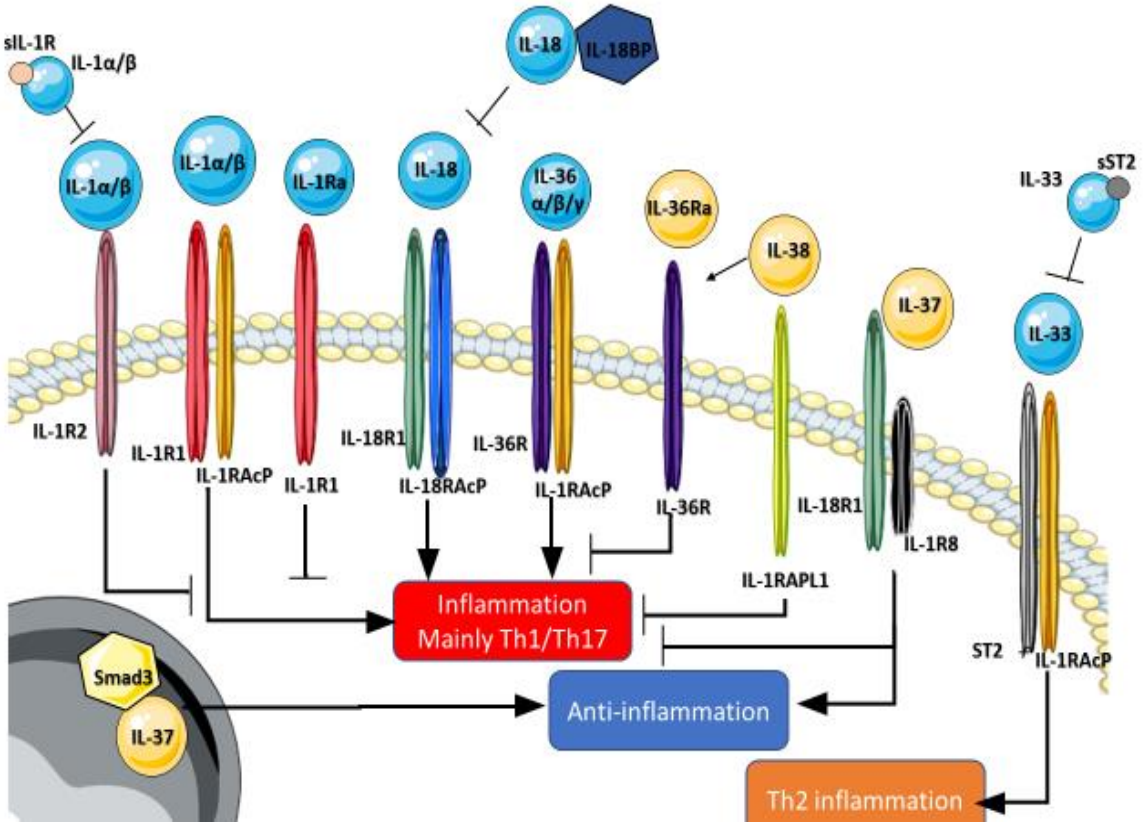



**Figure 1.3 IL1 family members and their receptors**

(Taken from Dinarello, C.A., 2019. The IL-1 family of cytokines and receptors in rheumatic diseases. *Nature Reviews Rheumatology*, 15(10), pp.612-632.)

| Family name | Name | Receptor/ coreceptor | Property | Synthesized as precursor | Processing required for activity |
|---|---|---|---|---|---|
| IL-1F1 | IL-1α | IL-1RI<br>IL-1RAcP | Pro-inflammatory | Yes | No |
| IL-1F2 | IL-1β | IL-1RI<br>IL-1RAcP | Pro-inflammatory | Yes | Yes |
| IL-1F3 | IL-1Ra | IL-1RI | Antagonist for IL-1α, β | No | No |
| IL-1F4 | IL-18 | L-18Rα<br>IL-18Rβ | Pro-inflammatory | Yes | Yes |
| IL-1F5 | IL-36Ra | IL-1Rrp2 | Antagonist for IL-36 | Yes | Yes |
| IL-1F6 | IL-36α | IL-1Rrp2<br>IL-1RAcP | Pro-inflammatory | Yes | Yes |
| IL-1F7 | IL-37 | IL-18Rα<br>IL-1R8 | Anti-inflammatory | Yes | Yes |
| IL-1F8 | IL-36β | IL-1Rrp2<br>IL-1RAcP | Pro-inflammatory | Yes | Yes |
| IL-1F9 | IL-36γ | IL-1Rrp2<br>IL-1RAcP | Pro-inflammatory | Yes | Yes |
| IL-1F10 | IL-38 | IL-1Rrp2 | ? Antagonist | Yes | ? No |
| IL-1F11 | IL-33 | ST2<br>IL-1RAcP | Pro-inflammatory | Yes | No |

**Table 1.2 IL-1 family members**

(Adapted from Carta, S., Lavieri, R. and Rubartelli, A. (2013). Different Members of the IL-1 Family Come Out in Different Ways: DAMPs vs. Cytokines? *Frontiers in Immunology*, 4.)

### 1.2.2 IL-37 Introduction

#### 1.2.2.1 IL-37

Interleukin-37 is a novel member of the IL-1 superfamily, previously termed IL-1 family member 7, IL-1F7, IL-1H, IL-1H4, or IL-1RP1. It was originally identified in 2000 (Busfield *et al.*, 2000; Kumar *et al.,* 2002). The Interleukin-1 (IL-1) superfamily includes 11 cytokines which share a ß-barrel structure and are closely located on human chromosome 2, except for IL-18 (chromosome 11) and IL-33 (chromosome 9) (Boraschi *et al.*, 2011). Functionally, most of IL-1 family cytokines, for instance, IL-1α, IL-1ß and IL-33 have pro-inflammatory functions and are produced in response to infection by pathogens, tissue damage and a variety of stimulatory molecules including other cytokines (Krumm *et al.*, 2014).

Several biological features of IL-37 are different from other IL-1 superfamily members. To be more specific, the majority of members of the IL-1 family are pro-inflammatory such as IL-1α and IL-18 (Nold *et al.*, 2013). In contrast, the most significant characteristic of IL-37 is suppressing the production and function of pro-inflammatory cytokines (Nold-Petry *et al., 2015*). During the inflammatory process, the synthesis of IL-37 is induced and activated similar to other IL-1 superfamily cytokines, but may mediate the production of other cytokines e.g. IL-6 and suppress the inflammation process. According to several reports, IL-37 acts as a key factor in regulating inflammation and has become an important inhibitor of innate immunity and inflammation in various diseases; for instance, colitis, LPS-induced septic shock and obesity-induced insulin resistance (McNamee *et al*, 2011; Nold *et al.*, 2010). This important regulatory characteristic of IL-37 is of particular interest.

#### 1.2.2.1 The IL-37 gene

The locus of the IL-1 cytokine genes is on human chromosome 2. Nine of the 11 IL-1 family member genes, including IL-37, map to 2q13 (Taylor *et al.*, 2002). However, IL-18 and IL-33 maps to 11 q22.2–q22.3 and 9 p24.1 respectively (Schmitz *et al.*, 2005). The location of the human IL-37 (IL1F7 gene) is between IL1B and IL1F9 genes. The size of IL1F7 is 3.617kb long, from the initiating methionine codon to the stop codon (Taylor *et al.*, 2002; Nicklin *et al.*, 2002).

IL-37 is the only member of the IL-1 superfamily for which there is as yet no identified mouse homolog. The explanation is that there is no known IL-37 gene in the mouse (Nold-Petry *et al.*, 2015). It is possible that the was an ancient IL-37 gene in the mouse which was lost through their evolution. There is evidence for the first exon of IL-37 gene in the mouse but without any open reading frame. Investigations for other IL-37 exons in the mouse have been unproductive. To explain this, it is possible that an ancient IL-37 in the mouse was disrupted by an insertion of endogenous retroviruses (Dinarello *et al.*, 2016). The expression of IL-37 protein is a major difference in immune potential between human and mouse, and this may provide an opportunity to investigate its function.

There are five splices of the human IL37 gene (IL-1F7). In these splice variants, three of contain the ß-trefoil unit, encoded by exon 4, 5 and 6, and are called IL-37 a, b and d (Dinarello *et al.*, 2010). In these variant transcripts, a consensus sequence of the IL-1 family has been detected in IL-37a, located at exon 4. Moreover, lysine 27, which contains the putative N-terminus, is situated 9 amino acids before this consensus sequence; and this putative N-terminus is essential for the activity of recombinant IL-37a. Although the activity of IL-37b with a N-terminus at lysine 53 has yet to be found, the bioactivity of IL-37b with N-terminus at valine 46 has been detected and confirmed; the recombinant form of IL-37b shows a high bioactivity (Li *et al.*, 2015). Another form of recombinant IL-37b which has a N-terminus at D20, binds weakly to IL-18Rα which is one of the two IL-37 receptors (Kumar *et al.*, 2002). There is currently no research finding describing the activity of recombinant IL-37c, d, and e.

Apart from the 12 putative ß-strands peptides encoded by IL-F7 which can form the ß-trefoil structure, IL-37 (IL-37b) also forms a homodimer under experimental conditions. Several studies reveal the existence of two single nucleotide polymorphisms in IL-37b; Glycine 31 to Valine and Threonine 42 to Alanine. In addition, the second methionine of exon 1 forms the IL-37b protein initiation expression site. This suggests that the sequence between Me 1 and Me 2 is essential for IL-37 (IL-37b) expression (Pan *et al.*, 2001). IL-37 cytokine is initially expressed in an immature form, or pro-protein, then the pro-protein actives externally to promote the development from an immature to mature IL-37 protein (Nold-Petry *et al.*, 2015). This activation is done by caspase activity. A Caspase-1 cleavage site has been detected in four of the IL-37 isoforms (isoform b-e), on exon 1 between D20 and E21 (A00492). Moreover, a second caspase-1 cleavage site exists on exon 2 between F45 and V46. In CHO cells, after the over-expression of IL-37 proteins, mature IL-37 protein (translated from V46) are detected (Boraschi *et al.*, 2011). Although IL-37a lacks exon 1, it has a unique exon 3, which has another putative elastase cleavage site between L21 and R22. Furthermore, there is a nuclear localisation signal site (NLS) on exon 3 at the N-terminus, upstream of the ß-trefoil structure, which assists the translocation of IL-37a into the cell nucleus and carry out their transcription factor functions (Nold-Petry *et al.*, 2015).

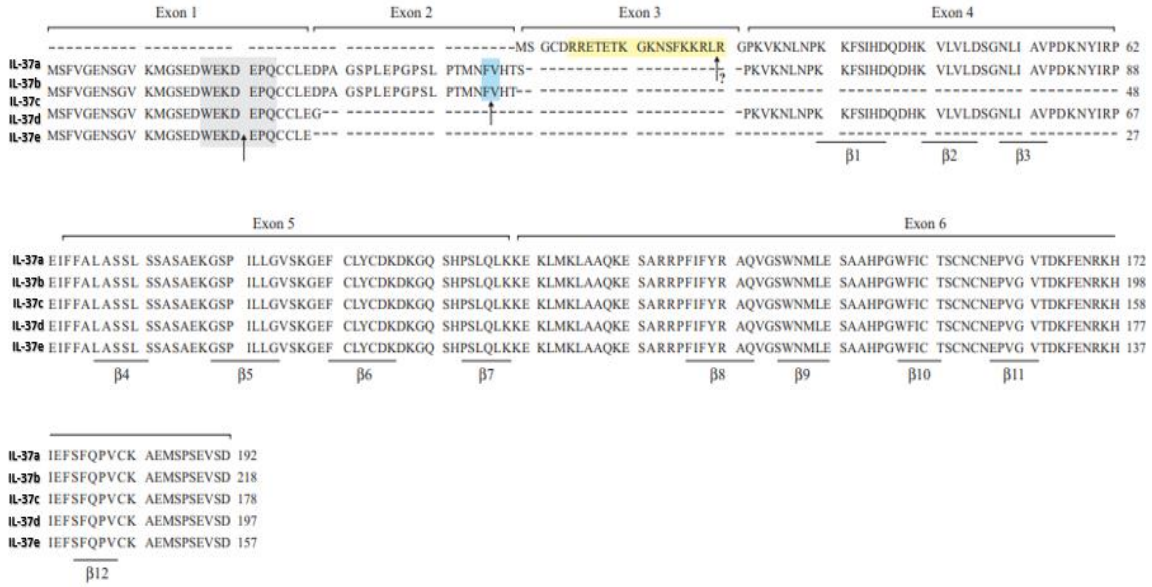



**Figure 1.4 Alignment of the predicted amino acids sequences of the five IL-37 isoforms**

Sequences of five IL-37 isoforms are detected by the free-ware system: 'ClustalW' (www.ebi.ac.uk/clustalw/). According to the arrangement of the six exons in the gene, all the exon positions are shown in the diagram above (Figure 1.4). Underlined amino acid alignment shows the position of ß-strands predicted by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). The black arrows mark the pro-peptide cleavage sites in this gene, with the grey shaded area on exon 1 between D20 and E21 being the predicted caspase-1 cleavage sites (exist in IL-37 b-e). As the authors indicate, there is another cleavage site which has been experimentally detected between F45 and V46 on exon 2 (marked by blue; present in isoforms b and c). Moreover, the black arrow with a question mark shows the location of the unique elastase cleavage site in exon 3, located between L21 and R22, and is only present in IL-37a. Apart from this unique cleavage site, IL-37a also contains a putative, bipartite nuclear localisation signal (NLS) detected by ProSite (http://www.expasy.ch/prosite/), spanning residues R5 to R22 (highlighted in yellow).

(Adapted from Boraschi, D., Lucchesi, D., Hainzl, S., Leitner, M., Maier, E., Mangelberger, D., Oostingh, G., Pfaller, T., Pixner, C., Posselt, G., Italiani, P., Nold, M., Nold-Petry, C., Bufler, P., Dinarello, C. (2011). IL-37: a new anti-inflammatory cytokine of the IL-1 family. *Eur Cytokine Netw*, 22(3), pp. 127-47).

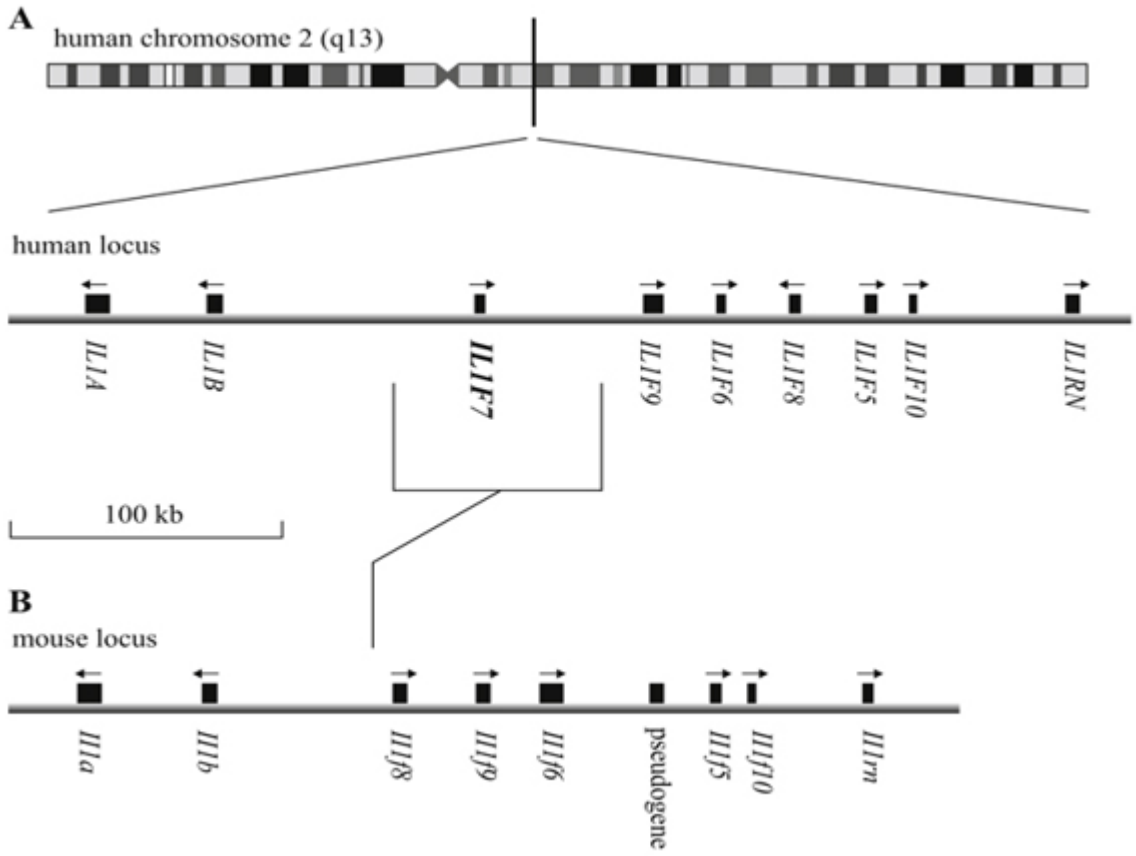



**Figure 1.5 Genomic location of IL-37 gene in human and mouse**

A) The IL-1 family gene locus on human chromosome 2. B) The IL-1 family gene locus on mouse chromosome 2. The IL-37 (IL-1F7) gene location between IL-1ß and IL-1F9 in human which is absent in mice.

(Adapted from Boraschi, D., Lucchesi, D., Hainzl, S., Leitner, M., Maier, E., Mangelberger, D., Oostingh, G., Pfaller, T., Pixner, C., Posselt, G., Italiani, P., Nold, M., Nold-Petry, C., Bufler, P., Dinarello, C. (2011). IL-37: a new anti-inflammatory cytokine of the IL-1 family. *Eur Cytokine Netw*, 22(3), pp. 127-47).

#### 1.2.2.2 IL-37 isoforms

Previous research has found out several fundamental structures of IL-37 which are called the rudimentary domain structures. These unique protein structures divide IL-37 into 5 parts known as the five isoforms of IL-37 (IL-37a-e) (Boraschi *et al.*, 2011). These isoforms have unique structures that may result in different functions in the inflammatory cascade. Although existing studies do not yet clearly explain the mechanisms and functions of IL-37, and the difference between its five isoforms, the majority of cohort studies focus on the largest splice variant, the IL-37b (isoform 1, 218 amino acids). Isoform IL-37b is the most prevalent variant of IL-37, and is the most studied and best characterized of the isoforms (Ye *et al.*, 2014). The most complete exon of IL-37b's sits with a putative caspase-1 cleavage site and a second cleavage site which can best characterize the structure and function of IL-37. IL-37a lacks exon 1 which is a common structure in the other 4 isoforms of IL-37, but has a unique N-terminus, encoded by exon 3, which is not expressed in the other isoforms (Charles and Philip, 2013).These structural features may endow a differential bioactivity and immune function to IL-37a. Furthermore, other short isoforms IL-37c, d and e lack exons 4, 2 or both (Boraschi *et al., 2011*).

IL-37 isoforms have a similar maturation step as the other members of the IL-1 superfamily. They are produced as precursor molecules. These pro-IL-37 molecules contain an enzyme cleavage site at their N-terminus. These are cleaved to become mature proteins (Taylor *et al.*, 2002). The expression of caspase-1 is required to produce IL-37 isoforms (except IL-37a) (Kumar *et al.*, 2002). However, research has shown that caspase-1 expression may not generate the complete production of IL-37b and IL-37c because of the other cleavage site (secondary cleavage site) which is encoded by exon 2 of the same gene (Taylor *et al.*, 2002). One possible implication of this is that the inhibition process of caspase-1 only partially influence IL-37b production, which indicates that there may be other cleavage sites apart from caspase-1 (Sharma *et al.*, 2008). The *in vitro* result of IL-37b maturation by Granzyme B and caspases 1 to 10 showed that only caspase-1 and 4 have the capacity to assist protein maturation. Moreover, caspase-1 cleavage was the most efficient of all the enzymes tested, with significantly lower

protein maturation rates observed using caspase-4, and no activity with other enzymes (Kumar *et al.*, 2002).

IL-37a has unique N-terminus encoded by exon 3, and a stop codon directly upstream of the starting methionine. The base sequence of the IL-37a gene is unlike a classic signalling peptide, but may act as a pro-domain which assists the maturation of the protein (Taylor *et al.*, 2002). Exon 3 is not present in other IL-37 isoforms, therefore the protein translation begins from exon 1. Moreover, IL-37a contains the same ß-trefoil structure (12 putative ß-strands, encoded by exon 4-6) as other members in IL-1 family. This suggests that IL-37a may be a cytokine with a biological function in the immune system (Boraschi *et al.*, 2011). Furthermore, there is a putative elastase cleavage site found between site L21 and R22. This unique feature not only reduces the requirement of caspase-1, a pre-requisite for IL-37a protein processing, but enables the pro-protein of IL-37a cleavage by elastase (Taylor *et al.*, 2002). Using an amino acid sequence alignment test, the length of the IL-37a protein is 172 amino acids (Taylor *et al.*, 2002).

IL-37c is an isoform of IL-37 which has a similar structure as IL-37b, but lacks exon 4. Both IL-37c and IL-37e lack the full sequence of 12-putative ß-strands encoded by exons 4-6. Due to the essential role of the complete sequence in protein folding and protein function, the lack of exon 4 in mRNA may cause aberrant protein-folding. Therefore, owing to the lack of this ß-trefoil unit, IL-37c and IL-37e may not have cytokine function. This means that both isoforms are unlikely fold into proteins which have the IL-1 superfamily 3D structure and may not bind to the IL-18 receptor (Boraschi *et al.*, 2011). Furthermore, although IL-37c and e are non-functional, they may act as regulatory factors for IL-37 protein production by competitively binding to the cleavage enzymes. Due to these enzymes being essential for pro-protein activation and following maturation, IL-37c and e may affect the expression of other isoforms (Boraschi *et al.*, 2011).

A variety of haematopoietic and non-haematopoietic cells can express IL-37, such as macrophages, monocytes, and neoplastic cells (Huang *et al.*, 2015). However, in several tissues, only one of the 5 isoforms of IL-37 is expressed: for instance, in the brain, only IL-37a has been detected, and IL-37b is the only isoform y expressed in the kidney, and IL-37c is the only isoform found in the heart (Boraschi *et al.*, 2011). According to the research of Dr Boraschi *et al.* (2011), the

constitutive expression level of IL-37 is low in the human body, and increased production requires stimulation. The reason for low expression levels is an instability component encoded by IL1F7 gene exon 5 that leads to strict regulation of IL-37 mRNA and subsequent reduced translation rates of IL-37 protein. In contrast, following LPS stimulation there is up-regulation of transcription of IL-37 with greater mRNA stability, and increased protein production (Borachi *et al.*, 2011). IL-37 mRNA stability is controlled by instability elements present in exon 5, the deletion of exon 5 can significantly increase mRNA stability of both IL-37b and IL-37c. Since all IL-37 isoforms contain exon 5, this instability element exists in all isoforms, to ensure an increase in mRNA stability only in inflammatory conditions. A similar mechanism can be observed for IL-18 (Bufler *et al.*, 2004).

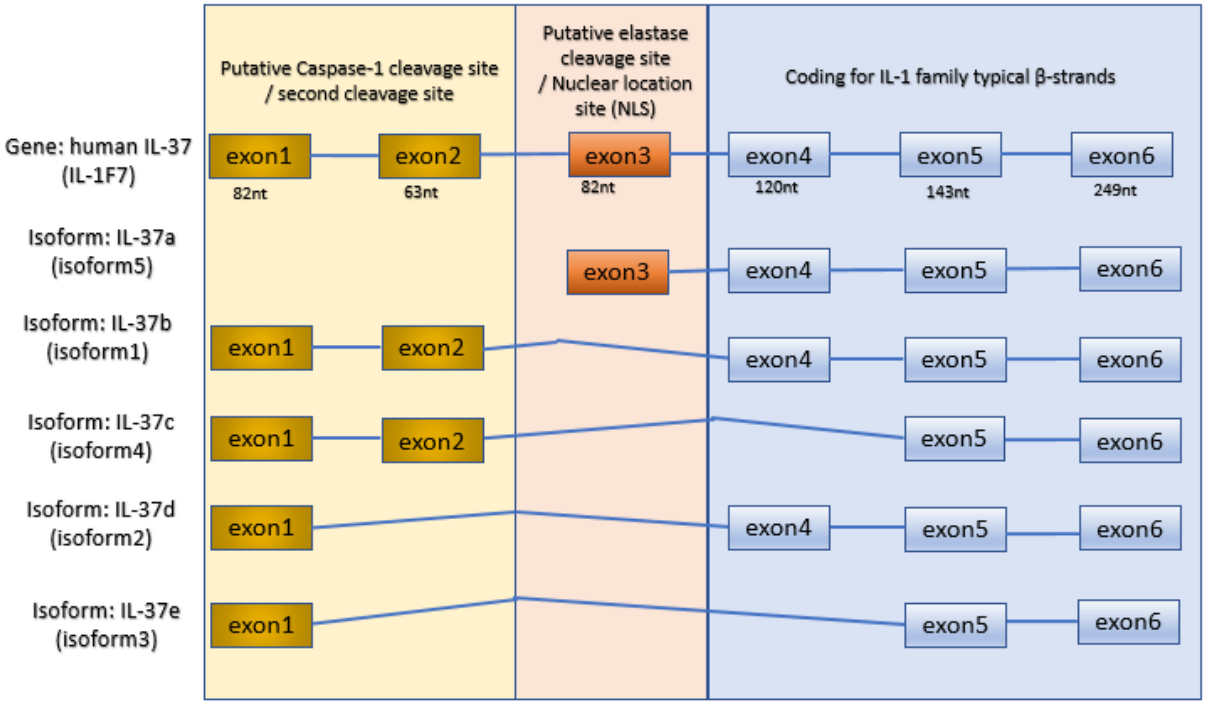



**Figure 1.6 Alternative splice variants showing the exon-intron interactions within the human IL37 gene, and the exon arrangements of the 5 isoforms of IL-37**

(Adapted from Boraschi, D., Lucchesi, D., Hainzl, S., Leitner, M., Maier, E., Mangelberger, D., Oostingh, G., Pfaller, T., Pixner, C., Posselt, G., Italiani, P., Nold, M., Nold-Petry, C., Bufler, P., Dinarello, C. (2011). IL-37: a new anti-inflammatory cytokine of the IL-1 family. Eur Cytokine Netw, 22(3), pp. 127-47).

### 1.2.2.3 IL-37 protein

With the help of immunohistochemistry, IL-37 protein can be detected in several human tissues and cells. This protein is expressed by plasma cells, blood monocytes, tonsillar B cells, neoplastic cells, epithelial cells and peripheral blood mononuclear cells (PBMCs) in many tissues. The expression of IL-37 has been demonstrated in a variety of cells including macrophages, monocytes, and neoplastic cells (Huang *et al.*, 2015). According to Boraschi *et al.* (2011), the level of IL-37 expression is low without stimulation. The reason is that an instability component encoded by IL1F7 gene exon 5 leads to strict regulation of IL-37 mRNA and this causes the low expression rates of IL-37 protein production. In contrast, stimulation by LPS can cause up-regulation of IL-37 protein production and higher mRNA stability.

As with other IL-1 family members, the precursor form of IL-37 protein does not contain any classical signal peptides. Pre-IL-37 can be detected by Western blotting technique in human blood monocytes stimulated by LPS. The constitutive concentration of IL-37 in serum is low (typically less than 100pg/ml) in healthy individuals. This concentration is increased during several diseases, for example, pre-eclampsia, lupus and rheumatoid arthritis (Southcombe *et al.*, 2015; Ye *et al.*, 2014; Zhao *et al.*, 2014). In human blood monocytes stimulated by LPS, additional exogenous ATP cause a modest release of processed IL-37; but the majority of IL-37 proteins are still in the precursor form (Bulau *et al.*, 2014). This illustrated that the additional ATP can active caspase-1 via NLRP3. This enhances the secretion of the IL-37 precursor, but it cannot significantly enhance the secretion of the processed IL-37 (Bulau *et al.*, 2014). Moreover, as with other IL-1 family members, such as IL-1α and IL-33, both precursor and processed IL-37 protein are biologically active (Kumar *et al.*, 2002; Li *et al.*, 2015). The mechanism by which the precursor IL-37 is processed to the mature form by proteases in the extracellular condition remains unclear.

According to the research of Ellisdon (2017), the IL-37b protein crystal stucture indicated that IL-37b can form a homodimer, which is a unique configuration in cytokines of the IL-1 superfamily. They confirmed that both the proIL-37(1-218) and the mature-IL-37(21-218), or IL-37(46-218) proteins can form homodimers

under certain conditions. There are several amino acids involved in the formation of dimers. The main amino acid which causes IL-37b monomers to form dimers involves the formation of hydrogen bonds between Tyr85 and Arg87. Moreover, this interaction is sealed with an ionic gate provided by another two amino acids Lys83 and Asp73 (Ellisdon *et al.*, 2017). Due to the lack of strands ß1 to ß3, the Isoforms IL-37c and e do not have the amino acids for dimerization (Ellisdon *et al.*, 2017).

The formation of homodimers may be enhanced when IL-37 is in high concentration, for example >1.35uM (Ellisdon *et al.*, 2017). Other factors e.g. pH and buffer composition have additional minor effects. Several research groups confirmed that the homodimerization of IL-37 can significantly reduce its immune suppressive function both *in vitro* and *in vivo*. The monomer of IL-37 is the active form for immune suppression (Eisenmesser *et al.*, 2019, Ellisdon *et al.*, 2017) although the detailed mechanism behind this is not fully understood, but it might represent a novel self-regulation of IL-37 function. This phenomenon along with the instability element of IL-37 RNA, suggest that the expression and activity of IL-37 can be highly self-regulated.

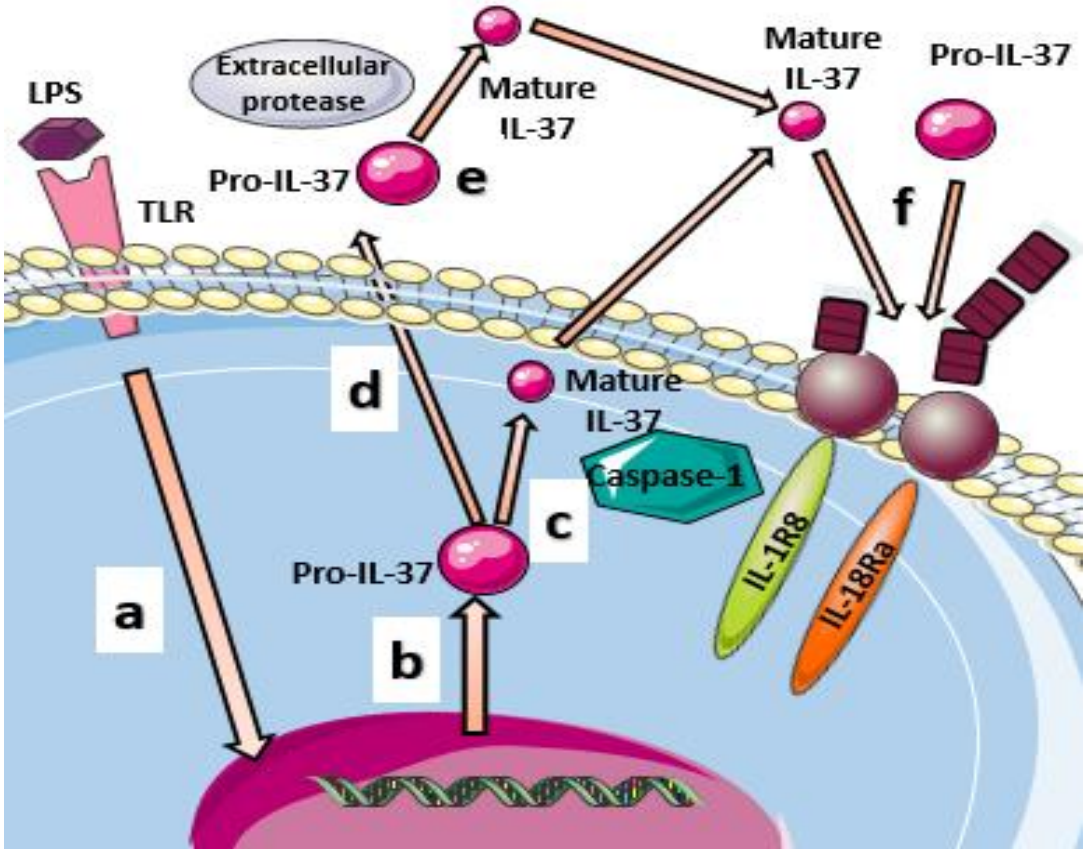



**Figure 1.7 IL-37 protein induction (TLR) and maturation**

(a) LPS activate immune cells which express IL-37. (b) IL-37 expression enhances and releases into the cytoplasm as pro-IL-37 after stimulation. (c) Caspase-1 enzyme catalyses pro-IL-37 into mature IL-37. (d) Mature and pro-IL-37 can secret into the extracellular space. (e) pro-IL-37 can be catalysed by extracellular protease into mature IL-37. (f) Both mature and pro-IL-37 can bind to IL-37 receptor complex and trigger signalling.

(Modified from Dinarello, A., Nold-Petry, C., Nold, M., Fujita, M., Li, S., Kim, S., Bufler, P. (2016). Suppression of innate inflammation and immunity by interleukin-37. *Eur J Immunol,* 46(5), pp.1067-81)

### 1.2.3 IL-37 signalling

IL-37 is thought to be a dual functional cytokine which can signal via receptor dependent and independent mechanisms (Dinarello *et al.,* 2016).

#### 1.2.3.1 IL-37 receptors

IL-37 has two receptors, IL-18Ra and IL-1R8.

##### 1.2.3.1.1 IL-18Ra

There are two receptors of IL-18; IL-18Rα and IL-18Rß. This is similar to IL-1α binding to its receptor IL-1RI and then recruiting IL-1RAcP to transfer the signal to activate the immune response. Initially, IL-18 binds to the receptor IL-18Rα then recruits another accessory protein IL-18Rß as co-receptor to activate immune cells (Huang *et al.,* 2015). Moreover, this accessory protein is essential to stabilize the interaction and forms a high-affinity (0.4nM) complex with biological function (Bufler *et al.,* 2004, Huang *et al.,* 2015). Due to the similar amino acid structure between IL-37 and IL-18, e.g. the Glu and Lys sites are the same, IL-37 can bind to the IL-18Ra (Bufler *et al.,* 2002; Sharma *et al.,* 2008). However, this binding of IL-37 to IL-18Ra is not sufficient to activate intracellular signal transduction; another receptor SIGIRR (IL-1R8) is recruited to form the tripartite complex and produce signal transduction of IL-37 (Bufler *et al.,* 2002; Garlanda *et al.,* 2009; Quirk *et al.,* 2014; Nold-Petry *et al.,* 2015).

#### 1.2.3.2 IL-1R8 (Single Ig IL-1-related receptor; SIGIRR)

IL-1R8 is an orphan receptor of the IL-1R family (Garlanda *et al.,* 2009). It is widely expressed in many human and mouse tissues, including liver, lung, kidney and intestine (Lech *et al.,* 2007). Moreover, it can be detected in B cells, macrophages, monocytes, dendritic cells (DCs) and natural killer cells (Lech *et al.,* 2007). IL-1R8 lacks the Ser447 and Tyr536 on the TIR domain, which is important for IL-1R1 signalling in other IL-1R family members. This feature of IL-1R8 structure means it can act as a decoy receptor which can down-regulate inflammation (Garlanda *et al.,* 2009).

IL-1R8 was believed to be a key negative regulator of IL-1 signalling, which orchestrated the differentiation and function of innate and adaptive lymphoid cells. Within the IL-1R8-deficient T cells, the IL-1 signal pathway was demonstrated to be enhanced compared to the wild type cells (Garlanda *et al.*, 2013). However, such inhibitory mechanism remained unclear. Following the engagement of IL-1 and the formation of hetero-dimer IL-1R1/IL-1RAcP, the recruitment of TIR domain-containing adapter proteins, such as MyD88 and MAL, was known to be essential in activating the IL-1 signalling. Striking, IL-1R8 was shown to be able to directly interact with some of these TIR domain-containing proteins, such as MAL, TRAM and TRIF (Qin *et al.,* 2005). Such interaction could further prevent the protein dimerization and signalosome formation, further dampening the downstream signalling. IL-1R8 exerts its regulatory activity by inhibiting NFκB and JNK activation induced by TIR-containing ILRs or TLRs upon ligand binding (Wald *et al.,* 2003). Because the TIR domain of IL-1R8 is altered (also named as TIRb), MyD88 binds to this TIRb domain resulting in a reduced signal to the nucleus (Garlanda *et al.,* 2013). Therefore, IL1R8 can modulate IL-1 signalling (Xu *et al.*,2015). The immune regulation function of IL-1R8 also associated with IL-37 function.

#### 1.2.3.3 IL-37 receptor-dependent signalling

A recent study identified a co-receptor system for IL-37b, involving IL1R8 and IL-18Ra (Nold *et al.,* 2013). Due to sharing similar critical amino acid residues with IL-18, IL-37 can bind to the IL-18 receptor IL-18Ra. However, compared with IL-18, the binding of IL-37 to IL-18Ra has low affinity and cannot become a competitive inhibitor (Boraschi *et al.,* 2011). The co-receptor system-IL-18Ra/SIGIRR may work as the anti-inflammatory signalling accessory chain, which assists the signalling process of anti-inflammatory proteins after binding to IL-37. The increased level of IL-37 in activated macrophages is associated with downregulation of inflammatory stimulation and a decreased response to the production of pro-inflammatory cytokines (Ballak *et al.,* 2014).

Both IL18Rα and IL-1R8 (SIGIRR) is essential in IL-37 protein function. In IL-37-transfected macrophages, there is an 83% reduction in IL-1ß level compare with un-transfected control macrophages. However, after silencing endogenous IL-1R8, only 34% of the reduction of IL-1ß production is observed. Silencing IL-18Rα also

causes an attenuation of the IL-37 effect (Nold-Petry *et al.*, 2015). Overall, these results indicate that IL-37 function is associated with IL-18Ra and IL-1R8; IL-1R8 is a co-receptor for IL-18Rα, and both receptors on the cell surface can bind to IL-37 and initiate an anti-inflammatory response following IL-1ß or LPS stimulation (Nold-Petry *et al.*, 2015). This interaction is essential for restraining inflammatory pathways in intracellular environment, for example, IL-37 and its co-receptors not only inhibit NF-κB and MAPKs, but also reveal pseudo-starvational effects on the mTOR pathway, inhibit TAK1 and Fyn and decrease the activation of the anti-inflammatory Mer, STAT3 and PTEN (Dinarello *et al.*, 2016). If one of the receptor complexes is missing, the effect of IL-37 will be attenuated.

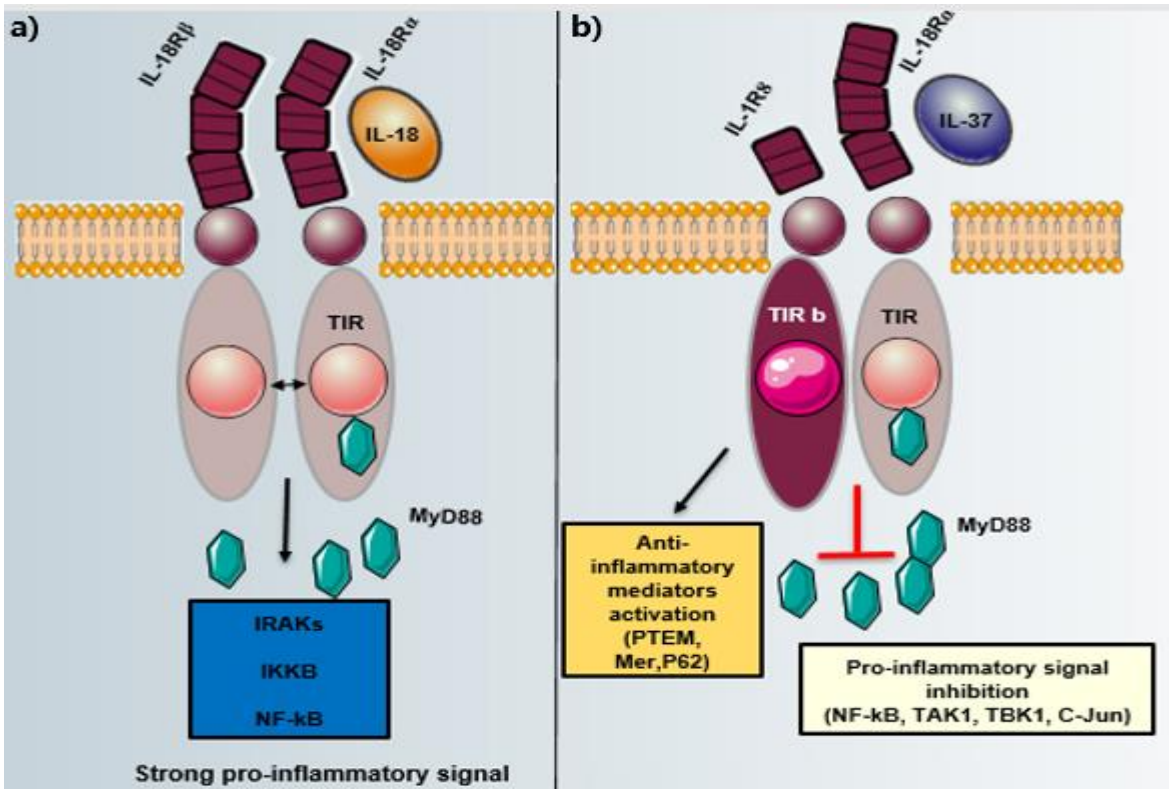



**Figure 1.8 IL-37 receptor-dependent signalling pathway**

Figure 1.8 shows the IL-37 receptor-dependent signal.

a) The classical pathway of IL-18 receptors.

b) IL-37 receptor dependent pathway. IL-37 protein binds to IL-18Ra first, recruit IL-1R8 and triggers anti-inflammatory signalling.

#### 1.2.3.4 IL-37 receptor-independent signalling

Another key characteristic of IL-37 is its regulation of inflammatory gene expression by a receptor-independent signalling pathway. Two isoforms of IL-37 (IL-37b and d) can suppress pro-inflammatory gene expression in immune cells that lack IL-37 receptors (Luo *et al.,* 2017; Zhao *et al.,* 2018). The mechanisms for nuclear translocation by this method remain unclear. The most-studied isoform IL-37b can translocate into the nucleus dependent on activation by capase-1, samd3 and the mature form of IL-37b (Zhao *et al.,* 2018). This can be confirmed by the following evidence: 1. Caspase-1 inhibition prevents IL-37 maturation, nuclear translocation, and reducing IL-37 function. 2. Mutation in the caspase-1 cleavage site (D20) inhibits IL-37b translocation into the nucleus (Bulau *et al.,* 2014). 3. Smad3-inhibitor inhibits IL-37 nuclear translocation, which can decrease inflammation *in vitro* and *in vivo* (Grimsby *et al.,* 2004; Bulau *et al.,* 2014; Dinarello *et al.,* 2016).

Therefore, IL-1 or TLR signals may elevate the IL-37 precursor synthesis in the cytoplasm. Furthermore, IL-1 or TLR can also induce the expression of caspase-1, which can catalyse pro-IL-37 into mature IL-37. The mature form of IL-37 can bind to phosphorylated-smad3 and form the IL-37-smad3 complex which can translocate into the nucleus. Although the detailed mechanism by which the smad3-IL-37 complex regulates gene expression in the nucleus remains unknown, due to the involvement of samd3 in TGFβ-mediated immunosuppression, IL-37 may also engage the TGF-β-smad3 signalling pathway to down-regulate inflammation (Grimsby *et al.,* 2004).

The pro-IL-37 protein (IL-37a with unique exon 3) can also translocate into the cell nucleus by its nuclear location site (NLS) on exon 3 (unpublished result). This observation provides a potential translocation function for IL-37a that is different from other isoforms. However, the effect of this translocation on inflammation is still unknown and needs further investigation.

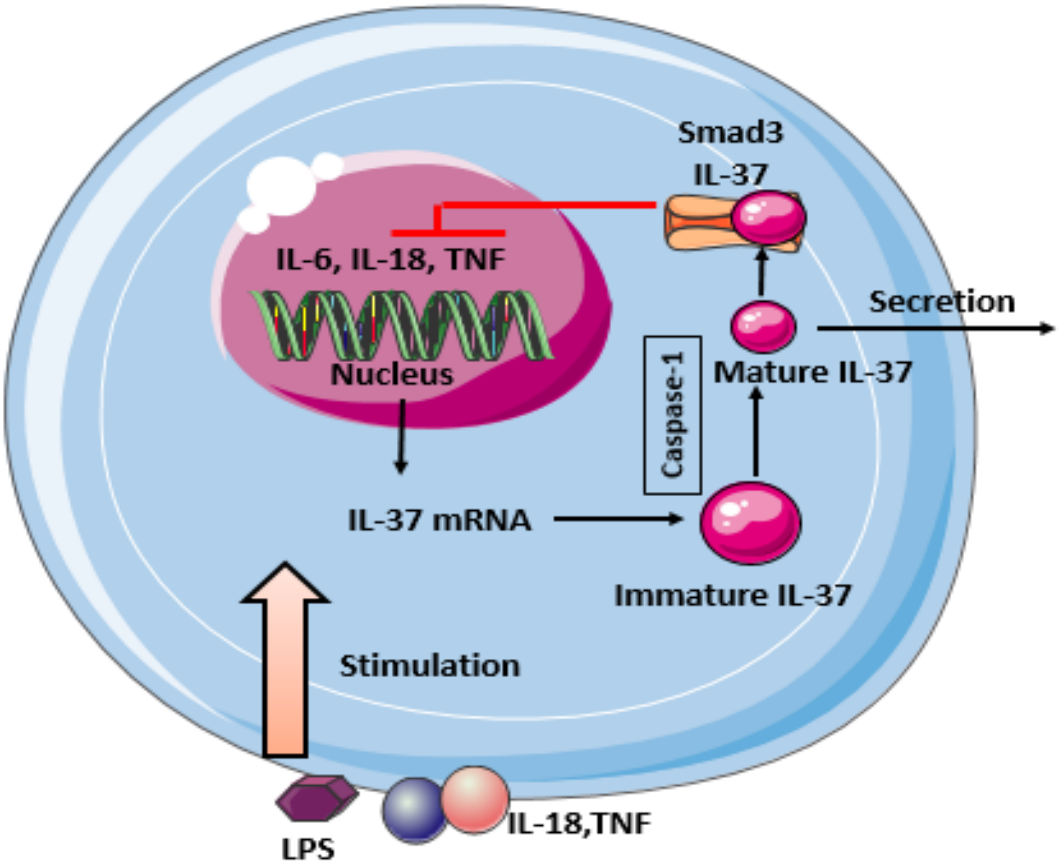



**Figure 1.9 IL-37 receptor independent pathway signalling**

### 1.2.3.5 IL-37 function in the immune system and disease

Previous studies that have noted the immune suppressive role of endogenous IL-37. For example, in human and mouse macrophages transfected with IL-37, the signalling kinases and production of IL-1ß-induced or LPS-induced cytokines are significantly reduced (Nold *et al.,* 2010). Moreover, in primary human blood monocytes, a knockdown of IL-37 causes the increased production of pro-inflammatory cytokines, such as, IL-1α, TNF-α, IL-1ß, IL-6, G-CSF, and GM-CSF (Nold *et al.,* 2010; Nold-Petry *et al.,* 2015). Furthermore, in human blood monocytes stimulated with LPS, a neutralizing antibody to IL-37 increases the production of pro-inflammatory cytokines (IL-1ß, IL-6, and TNF-α) (Li *et al.*, 2015). These observations support the concept that IL-37 can down-regulate the production of pro-inflammatory cytokines in primary human cells.

IL-37 can suppress the production of LPS-induced cytokines, transcription factors, and signalling kinases. The mouse macrophage cell line (RAW cells) was transformed with human IL-37b (the most studied and the longest isoform with 5 exons of IL-37). These transfected cells, which now constitutively expressed IL-37b, were stimulated with LPS and the production of pro-inflammatory cytokines and chemokines (including IL-1α, IL-1ß, IL-6, IL-23, TNF-α, GM-CSF and soluble ICAM-1) were significantly reduced (Nold *et al.,* 2010; Nold-Petry *et al.,* 2015, Sharma *et al.,* 2008). There were similar reductions in cytokine production by bone marrow cells of IL-37b transgenic mice. Various signalling phosphokinases were reduced in these cells, including mammalian target for rapamycin (mTOR), FAK, p53, STAT1, STAT3, Syk, p38, paxillin, and SHP-2. In contrast, the anti-inflammatory pathways were elevated in the IL-37Tg mouse. For example, the kinase and the induction of the phosphatase PTEN was enhanced in comparison with wild-type mouse cells (Nold-Petry *et al.,* 2015, Sharma *et al.,* 2008). PTEN plays a crucial role in regulating by inhibiting pro-inflammatory pathways such as mTOR, FADK and MAPK (Dinarello *et al.,* 2015).

#### 1.2.3.5.1 IL-37 and Inflammatory Diseases

**IL-37 and Inflammatory Bowel Disease**

Gene expression of IL-37b is significantly increased in inflammatory bowel disease (IBD) patients compared with healthy control groups. The IL-37b is mainly produced in circulating B cells, activated NK cells and monocytes, and correlates with the severity of inflammation (Nold-Petry *et al.*, 2015). This indicates that IL-37 may precipitate in homeostati attempts to control the reduction of inflammation in IBD patients (Imaeda *et al.*, 2013; Fonseca-Camarillo *et al.*, 2015; Li *et al.*, 2014). Furthermore, the IL-37Tg mice have lower clinical and histological scores compare with wild type mice in colitis models (McNamee *et al.*, 2011).

**IL-37 and Inflammatory Respiratory Diseases**

IL-37 levels in serum of asthma patients were lower than in healthy control subjects, and the levels correlated negatively with asthma severity (Charrad *et al.*, 2016). The IL-37 may act as an anti-inflammatory cytokine which can suppress the inflammation in allergic asthma by suppressing inflammatory cytokine (mainly Th2 cytokine) production. Moreover, in an asthmatic mouse model, IL37b can significantly reduce the allergic airway inflammation, the airway hyperresponsiveness, decreases the inflammatory cell infiltration into the lung tissue, and dampens Th2 cell function. Therefore, IL-37b participates in the pathogenesis of asthma (Lunding *et al.*, 2015). However, recombinant IL-37 does not seem to have immune suppressive function in receptor-knockout mice in an allergic asthma model, which indicate that the anti-inflammation function of IL-37 is dependent on the receptor IL-18Rα and SIGIRR/IL-1R8 (Lunding *et al.*, 2015). Furthermore, the IL-37 level and the inflammasome inhibitory molecules NALP7 are increased in the bronchial mucosa of stable chronic obstructive pulmonary disease (COPD) patients compared with control smokers (Di *et al.*, 2014).

**IL-37 and Atherosclerosis**

Serum IL-37 levels are elevated in atherosclerosis patients. The IL-37 cytokine produced by macrophage can reduce the production of pro-inflammatory cytokines (IL-1B, IL-6, and IL-12) *in vitro*, and reduces the development of atherosclerosis of patients (McCurdy *et al.*, 2017). Moreover, IL-37 is highly expressed in human atherosclerotic plaques, and ameliorates atherosclerosis formation by inducing Treg response (Ji *et al.*, 2017). Therefore, IL-37 may act as a potential therapeutic approach to prevent atherosclerosis formation and to treat disease.

#### 1.2.3.5.2 IL-37 and Autoimmune Diseases

**IL-37 and Graves' Disease**

IL-37 expression in patients with GD are significantly higher than healthy controls. In GD patients, IL-37 acts as an anti-inflammatory cytokine which protects against the inflammatory effect in GD by reducing the pro-inflammatory cytokines production, such as IL-6, IL-17 and TNF-α (Li *et al.*, 2014).

**IL-37 and Rheumatic Disease**

The expression of IL-37 is significantly augmented in rheumatoid arthritis (RA) patients compared with healthy controls. In murine arthritis models, injected recombinant IL-37b ameliorates the clinical symptoms and delayed disease development. The regulatory function of IL-37 in RA relates to the inhibition of Th17 cell proliferation, and the decreased pro-inflammatory cytokine production in synovial cells (such as IL-1b and IL-6) (Ye *et al.*, 2014). Moreover, IL-37 also affects the monosodium urate crystal-induced inflammation in a Mer receptor tyrosine kinase (Mertk)-dependent fashion (Liu *et al.*, 2016).

**IL-37 and Systemic Lupus Erythematosus**

Plasma IL-37 concentration in systemic lupus erythematosus (SLE) patients is higher than in healthy controls (Song *et al.*, 2013). The increased expression of IL-37 is associated with anti-RNP, anti-Sm and C3 levels in patients, and correlates with disease severity and renal damage (Tawfik *et al.*, 2017). Moreover, IL-37 can control the pathogenesis of SLE by reducing pro-inflammatory cytokine production (IL-1b, IL-6 and TNF-α) (Ye *et al.*, 2014).

**IL-37 and Psoriasis**

IL-37 is highly expressed in psoriatic skin biopsies and is mainly expressed by macrophages and effector memory T cells in human psoriatic plaques. Moreover, in human keratinocytes from psoriasis patients, the human beta-defensin-3 was shown to enhance IL-37 expression (Keermann *et al.,* 2015; Smithrithee *et al.,* 2015).

**IL-37 and Behcet's Disease**

In Behcet's disease (BD), IL-37 expression level was decreased and correlated with high inflammatory responses, for example, low IL-37 level is associated with the activation of Th1 and Th17 cells, and increases the production of inflammatory cytokines and reactive oxygen species (Bouali *et al.,* 2015). Corticosteroid treatment augmented IL-37 mRNA expression and this results in a reduction of inflammatory cytokines (Ye *et al.,* 2014).

**1.5.3.2.3 IL-37 and cancer**

IL-37 gene expression is increased in several cancers, and this may become a potential therapeutic target in cancer pathogenesis. The main role of IL-37 in cancer is a protective function. IL-37 has a therapeutic role in cancer development. Until now, there are no results that show that IL-37 can be a pro-tumour cytokine (Ding *et al.,* 2016). To be specific, IL-37b downregulates the STAT3 pathway in cervical cancer (CC) by inhibiting essential tumorigenic factor STAT3 expression and phosphorylation which may become a novel treatment strategy of CC (Wang *et al.,* 2015). IL-37 can also downregulate the STAT3 pathway in renal cancer (RC) and suppresses IL-6, HIF-α and Bcl2 expression. Moreover, IL-37 can suppress fibrosarcoma and non-small cell lung cancer but the mechanism remains unknown (Haabeth *et al.,* 2014). Therefore, IL-37 might be a novel therapeutic regulator in cancer.

#### 1.2.3.2.4 IL-37 function in infectious disease

Endotoxemia is a severe disease induce by LPS from gram-negative bacterial infection. It may cause endotoxin shock and is life threatening by causing multiple organ failure (Li *et al.*, 2015). The mechanism of septic shock is the over-activation of TLR4 signal which aberrantly elevates inflammatory cytokine production, such as IL-1, IL-6, TNF-a, and oxygen free radicals (Wang *et al.*, 2018). The down-regulatory function of IL-37, especially the isoform IL-37b in the mouse model of LPS-induced endotoxemia has been confirmed (Li *et al.*, 2015).

Endotoxemia is the best studied IL-37-regulated disease (Li *et al.*, 2015). The transgenic human IL-37b mouse has reduced tissue inflammation and inflammatory cytokine production in the LPS shock model, compare with wild-type mice (Bulau *et al.*, 2014). This regulatory function may be mediated by both the receptor-dependent and independent pathways. To confirm this, one RNA-seq assay was performed on IL-37b transgene mice cells with or without IL-1R8 *in vitro*. The results demonstrated:

i) Several genes of anti-inflammatory and metabolic mediators were activated by IL-37 in IL-1R8 dependent pathways, such as FOXO-1, PTEN and STAT3, and inhibit the metabolic checkpoint mTOR which downregulate some genes of inflammatory mediators like c-jun, IKß and TBK1; the IL-37 can also suppress the NF-kß pathway which is essential for inflammatory signalling.

ii) Apart from the receptor dependent pathway, IL-37 can regulate the LPS-induced transcription factors activity by receptor independent pathway, which can significantly suppress the inflammatory genes production, such as STAT5, SHP-2and IGF-1R. Therefore, the dual regulatory functions of IL-37 in receptor dependent and independent pathways of LPS-induced endotoxemia have been confirmed (Nold-Petry *et al.*, 2015).

IL-37 also participate in other infectious diseases:

**Infectious diseases**

Despite the successes of antibiotics and vaccination, pathogenic infection is still a big health problem. This is mainly due to the increasing antibiotic resistant bacteria and the emerging mutant or new strains of pathogens. Some pathogens can also evolutionarily develop the ability to help them to survive and colonise in the human environment by silencing host protective immune mechanisms, for instance the vIL-10 produced by EBV (Kanai *et al.*, 2007).

Immune system plays an essential part in protection against infection. As described in Chapter 1, during the early infection, innate immune cells, including macrophages, DC and neutrophils can be rapidly activated to control pathogen spread and colonisation in the host. Later, the adaptive immune cells, T and B cells will also be specifically activated, and can eliminate the pathogens by cytotoxicity (CD8 T cells), helping other cells (CD4+ T cells) and producing antibody (B cells). While a rapid and strong inflammatory response helps in pathogen clearance, host tissue destruction secondary to immunopathology can be a deleterious side effect. An appropriate anti-pathogenic response is therefore necessary for minimising tissue damage while still allowing clearance of the invading pathogen.

Regulatory cells and related cytokines are critically needed for this purpose to maintain host homeostasis and immune tolerance (Abbas *et al.*, 2012). The importance of IL-37 in infection has just emerged and available reports suggest that IL-37 plays a regulatory role in bacterial, virus, parasitic and fungus infections. However, its role in infection is variable depending on the pathogen.

**Viral infection**

It has been shown that IL-37 can influence the infection and pathogenesis of HIV, coxsackie virus B3 and influenza infection (Hojen *et al.,* 2015; An *et al*. 2017; Zhou *et al.,* 2018).

**Viral myocarditis**

Viral myocarditis is an inflammatory heart disease which is caused by many viruses, including Influenza, hepatitis A & C viruses, HIV and coxsackie virus B3 (CVB3) (Zhao *et al.,* 2018). CVB3 causes myocarditis in both human and in animal (Spartalis *et al.,* 2017). CVB3 infection elicits TLR4 signals in APCs that subsequently activates innate and virus-specific TH17 cells to enhance pro-inflammatory cytokine production and myocarditis (Fairweather *et al.,* 2005). It has been reported that IL-37 treatment impaired inflammation, pathology and reduced mortality in mouse model of myocarditis. This was accompanied by reduced effector function of Th17 cells but enhanced immunomodulatory Treg cell numbers in the spleen of myocarditis mice. Furthermore, IL-37 not only inhibits IL-6 and IL-17A, also promote IL-10 levels in the heart. These results suggest that IL-37 is potentially therapeutic. More clinical experiments using human cells/tissue are needed to confirm the hypothesis (An *et al.,* 2017).

**Influenza A virus (IAV) infection**

The expression and function of IL-37 in influenza A virus (IAV) infection was also studied (Zhou *et al.,* 2018). The levels of IL-37 mRNA and protein levels in the PBMC and serum samples from patients with influenza A virus (IAV) infection were enhanced compared to the healthy subjects. To understand the effect of IL-37 on IAV replication in epithelia cells, A549 cell line was infected with IAV first and then treated with rIL-37. The authors found that the level of IAV RNA was significantly reduced in IL-37 treated cells. The result suggested that IL-37 may be able to inhibit the replication of IAV RNA in infected cells. However, the mechanism underlying the IL-37-reduced virus replication has not been explored.

**Bacterial infection**

The role of IL-37 in mycobacterium tuberculosis (Mtb), Strongyloides stercoralis and pneumococcal pneumonia infection has been reported (Paterson and Orihuela, 2010; Liu *et al.,* 2017; Schauer *et al.,* 2017).

Tuberculosis (TB) is caused by Mycobacterium tuberculosis and affects one quarter of the world's population. M1 and Th1 cells that produce IFN-γ may play a protective effect on TB (Berry *et al.,* 2013) and M2 cells that produce immunoregulatory cytokines are thought to be pathogenic (Huang *et al.,* 2015).

Recent reports suggest that IL-37 may play a critical role in TB, however, the results are controversial in human and in mice (Huang *et al.,* 2015; Luo *et al.,* 2016). Zhang *et al.* reported that IL-37 level is increased in TB patients. The elevated IL-37 production in TB was positively associated with the prolonged/complicated TB and also the TB burden, while it is negatively correlated with the level of pro-inflammatory cytokines such as IL-1ß, IL-6 and TNF-α in the patients (Zhang *et al.,* 2016). The result suggests that il-37 may be pathogenic in TB by inhibiting protective inflammatory response thereby promoting infection.

In agreement with Zhang, Liu and colleagues further found that IL-37 single nucleotide polymorphism (SNP) is closely associated with TB susceptibility. The levels of IL-37 protein in TB patients were elevated compared to levels in healthy controls. Furthermore, Mtb infection enhanced IL-37 but reduced proinflammatory cytokines including IL-6 and TNF-α production in macrophages. However, they demonstrated that IL-37 may be protective against mycobacterial infection in mouse model *in vivo* (Liu *et al.,* 2017); BCG-infected IL-37Tg mice have less mycobacterial burden and tissue damage in the lung, compared to the infected WT control mice. This suggests that IL-37 can protect against Mtb infection in mice. Moreover, the immune mechanism study demonstrated that the protective effect of IL-37 was associated with an enhanced level of Th1 cells and reduced regulatory T cells in the spleen (Liu *et al.,* 2017). Thus, IL-37 may protect by promoting anti-bacterial Th1 response and downregulating disease-promoting T-reg response in

Mtb infection. Unfortunately, how IL-37 promotes Th1 and inhibits Treg response in Mtb infection is still unclear.

Streptococcus pneumonia (SP) infections can cause several human diseases, including pneumonia, bacteraemia, and meningitis by enhancing inflammatory pathological response (Paterson and Orihuela, 2010).

Given the effect of IL-37 as a suppressor of innate and acquired immunity, its impact on SP infection has been studied (Schauer *et al.*, 2017). *In vitro*, the overexpression of IL-37 in the macrophages infected with pneumococci decreased the levels of proinflammatory cytokines IL-6, TNF-α, and IL-1β, but increased the intracellular pneumococcal infection. Consistently, *in vivo*, compared to the WT control mice, infection with S. pneumonia in IL-37b transgenic mice also decreased the expression of inflammatory cytokines IL-6, TNF-α, and IL-1β in lung tissue at early infection. However, at later stage, IL-37Tg mice enhanced the pneumococcal burden, lung tissue inflammation, damage and mortality. Thus, this is consistent with our result in salmonella infection and suggests that IL-37 is a therapeutic target in SP-induced pneumonia and in salmonella infection.

**Helminth infection**

The role of IL-37 in parasite infection is less studied. Only two reports from same research group so far show that IL-37 can modulate T cell immune response and cytokine balance in Strongyloides stercoralis infection (Anuradha *et al.*, 2015).

Strongyloidiasis is a common parasitic disease which affects about 100 million people worldwide (Anuradha *et al.*, 2015; Anuradha *et al.*, 2017; Patton *et al.*, 2018). The protective immune mechanism in strongyloidiasis in human is largely unknown. The Th1 or Th17 response can be protective or pathogenic depending on the degree of T cell responses. Th2 cell-mediated immune response is thought to play a protective role in the strongyloidiasis by counter-regulation of Th1/Th17-mediated pro-inflammatory response (Anuradha *et al.*, 2015; Anuradha *et al.*, 2017; Patton *et al.*, 2018). It has been shown that the patients with strongyloidiasis infection contain significantly higher levels of serum proinflammatory cytokines IFN-γ, TNF-α, and IL-1β and significantly lower levels of anti-inflammatory cytokines IL-4, IL-10, IL-13, IL-27, IL-37, and TGF-β,

compared to healthy controls (Anuradha *et al.,* 2015). However, after treatment, the cytokine profiles can be reversed with negative enhanced levels of inflammatory cytokines and IL-1β but reduced anti-inflammatory cytokines including IL-37 (Anuradha *et al.*, 2015). To assess the role of IL-37 in the regulation of CD4+ and CD8+ T cell responses in strongyloidiasis, the authors measured the changes of parasite specific Th1/Tc1, Th2/Tc2, Th9/Tc9, Th17/Tc17, and Th22/Tc22 cell response in the PBMC from the patients with strongyloidiasis and healthy controls following the anti-IL-37 neutralizing antibody treatment *in vitro*. They found that IL-37 neutralization markedly increased the level of parasite-specific Th1/Tc1, Th2/Tc2, Th17/Tc17, Th9, and Th22 cells by FACS and related cytokines IFN-γ, IL-5, IL-9, IL-17, and IL-22 by ELISA in the cultures. Thus, while the reports demonstrate that IL-37 can modulate S. Stercoralis-specific T cell response, the precise role of IL-37 in the protective immunity against strongyloidiasis is still unclear (Anuradha *et al.,* 2017).

**Fungal infection**

There are only two detailed reports in PubMed so far that studied the potential role of IL-37 in fungal infection *in vitro* and *in vivo,* the candida and aspergillosis (Moretti S *et al.,* 2014; Veerdonk *et al.,* 2015).

Candidiasis is a disease of fungal infection. While the protective immunity against candida infection is still not fully understood, the innate immune cells, in particular the neutrophils in the mucosal tissue, play an important role in anti-candida infection (Anuradha *et al.,* 2017). Given its effect on the suppression of neutrophil migration and function, the authors proposed that IL-37 may promote infection by affecting neutrophils function (Van de Veerdonk *et al.,* 2015). They demonstrated the hypothesis in a murine model of disseminated candidiasis using IL-37b transgenic mice. Compared to the WT control mice, IL-37-tg mice were susceptible to candida infection, enhanced fungal growth in the organ and mortality. The mice also failed to effectively produce inflammatory cytokines and recruit neutrophils to the site of infection. Thus, IL-37 may be pathogenic in candidiasis by interfering with host innate protective inflammatory immune response. However, its effect in human candidiasis has not been explored.

**Aspergillosis**

Aspergillosis is caused by the aspergillus which is the most common fungus in the environment (Moretti *et al.,* 2014). Aspergillus infection can cause clinical conditions including opportunistic infection and allergy, depending on host immune state. (Kosmidis and Denning, 2015).

Activation of inflammasome during Aspergillus infection is pathogenic for promoting the secretion of IL- 1b and can result in pathological sequelae (Sun, 2018). In murine model of Aspergillus fumigatus, IL-37 plays an anti-inflammatory role to suppress IL-1 production via control of NLRP3 inflammasome activity. Furthermore, treatment of A. fumigatus infected mice with recombinant IL-37 also reduced lung inflammation and increased IL-10 production in lung tissue. Thus, IL-37 display therapeutic potential in decreasing the Aspergillus-induced pulmonary pathogenesis (Moretti *et al.,* 2014).

## 1.3 TLRs introduction

Toll-like receptors (TLRs) are a family of cell receptors that recognize conserved surface structures on pathogens (pattern recognition receptors PRRs). Currently, 11 and 13 types of TLRs have been identified in humans and mice, respectively. These TLRs widely exist on the cell membrane and intracellularly in many cell types, for example, macrophages, dendritic cells (DCs), neutrophils, and mucosal epithelial cells (Zhang *et al.,* 2015). Examples of microbial recognition include Gram negative endotoxin which is recognized by TLRs (particularly TLR4) and binding can promote neutrophil activation to enhance phagocytosis of the bacteria and their intracellular killing, and the production of anti-bacterial substance e.g. alpha-defensins. Microbial recognition by TLRs can also enhance the cytotoxicity of NK cells and maturation of DCs which is a link to adaptive immunity, antibody production, and costimulatory and T cell activation (Casals *et al.,* 2007). The persistence of infection, microbial recognition and binding to TLRs are one of the mechanisms by which the innate immune system in turn activates and maintains the adaptive immune response at the appropriate level required to match the immune challenge of the infection.

TLRs are a category of transmembrane Pathogen Recognition Receptors (PRRs) which can bind to Pathogen Associated Molecular Patterns (PAMPS) and then activate the cells. This activates intracellular signalling pathways e.g. the nuclear factor kappa-light-chain-enhancer of activated B cells pathway (NF-kB) (Medzhitov, 2001). Activated NF-kB can regulate DNA transcription and cell survival, and the synthesis and secretion of cytokines and other immune mediators which can activate other innate immune pathways and assist the signalling between immune cells to optimize their response (Beutler *et al.,* 2006).

To maintain virulence, pathogens have evolved various tactics during infection to subvert the host anti-microbial mechanisms. Genetic mutation of their antigenic structures e.g. in influenza virus can ensure their survival, replication, and persistence during infection (Coburn *et al.,* 2007). This is countered by the host profile of somatic line of immune system PRRs which recognize various microbial molecular patterns to elicit an appropriate and fast innate immune response to resist infection. This ongoing 'arms-race' interaction between the pathogen virulence factors and innate immune responses of host largely determines the outcome of the majority of infections (Zhang *et al.,* 2015).

### 1.3.1 TLRs signalling pathways

There are 5 steps associated with TLRs signalling:

1. Pathogen PAMPs interact with the LRR motif of TLRs to create complexes then induce TLRs dimerization.

2. Through the Toll/interleukin-1 receptor (TIR) domain of TLRs, the intracellular signalling adaptor molecules, e.g. MYD88 and the TIR homology domain (TRIF) recruit and transmit the activation signal.

3. Binding induces the production of protein kinases, e.g. Protein Kinases C (PKC).

4. These protein kinases recruit transcription factors which are reciprocally activated by phosphorylation and dephosphorylation.

5. The activated transcription factors translocate to the cell nucleus and bind to specific complementary gene sequences (response elements) which lead to

expressions of pro-inflammatory cytokines, chemokines and cell surface molecules (Medzhitov, 2001).

TLR signals can be divided into two main pathways depending on the participation of the adaptor molecule MyD88. The pathways can be either MyD88 dependent or independent. These MyD88 signalling pathways are essential in almost all examples of TLR and IL-1R signal transduction except TLR3.

**The MyD88-dependent pathway** is initiated by recruiting the adaptor molecule MyD88 following TLR binding to its specific ligands. Stimulation of MyD88 is followed by combination of the TIR domains with both MyD88 and IL-1R-associated kinase (IRAK-4) to create an immune complex termed as the "MyDdosome". IRAK-4 then phosphorylates IRAK-1 which recruits the TNF receptor-associated factor 6 (TRAF-6) which activates either TAB or TGF-ß activated kinase (TAK-1) complex. Formation of TAK-1/TAB complex increases the activity of the IκB kinase (IKK) complex which degrades Iκß33 and causes nuclear translocation of the transcription factor NF-κB. The NF-κB can induce the production of proinflammatory cytokines, such as IL-6, IL-12, TNF-α and other mediators (Wang *et al.,* 2001).

**The MyD88-independent pathway** (also called the TIR domain containing adaptor inducing IFN-ß (TRIF)-dependent pathway) is only used by intracellular TLR3or internalized TLR4 (Jiang and Chen, 2012). The activation starts with the stimulation signal of TLRs, which recruits TRIF and activates the transcription factor IRF-3. This transcription factor can translocate into the nucleus and restrict activation of NF-κB pathways. The MyD88-independent pathway can produce type I IFNs, then activate STAT1 and induce expression of IFN-inducible genes (Toshchakov *et al.,* 2002). Therefore, this pathway is essential for the innate response to viruses and intracellular infections by the production of type I IFNs.

As discussed above, TLRs provide a rapid innate response to pathogens. TLR activation can contribute to the host response to antigens and can provide a bridge linking the innate and adaptive immune response (Rolls *et al.,* 2007). For example, during salmonella infection, TLR activation by bacterial LPS can initiate the host inflammatory cell response by activating and recruiting macrophages and neutrophils to the site of infection, and then producing cytokines. These cytokines

include Interleukin (IL)-1ß, IL-6, interferon-gamma (IFN)-γ and tumor necrosis factor (TNF)-α (Mittrucker *et al.,* 2002). An important cytokine produced during salmonella infection is IFN-γ because this cytokine can by regulates the activation status of macrophage which can clear the infection (Percival *et al.,* 2012). IL-18- also plays a central role in assisting IFN-γ expression as well as early resistance_ of immune system towards infection of *S. Typhimurium*. This protective response can become pathogenic, for example, during an invasive salmonella infection and an aberrantly activated pro-inflammatory immune response may cause excessive inflammation, tissue damage, intravascular coagulation and potential death (Hanna *et al.,* 2012).

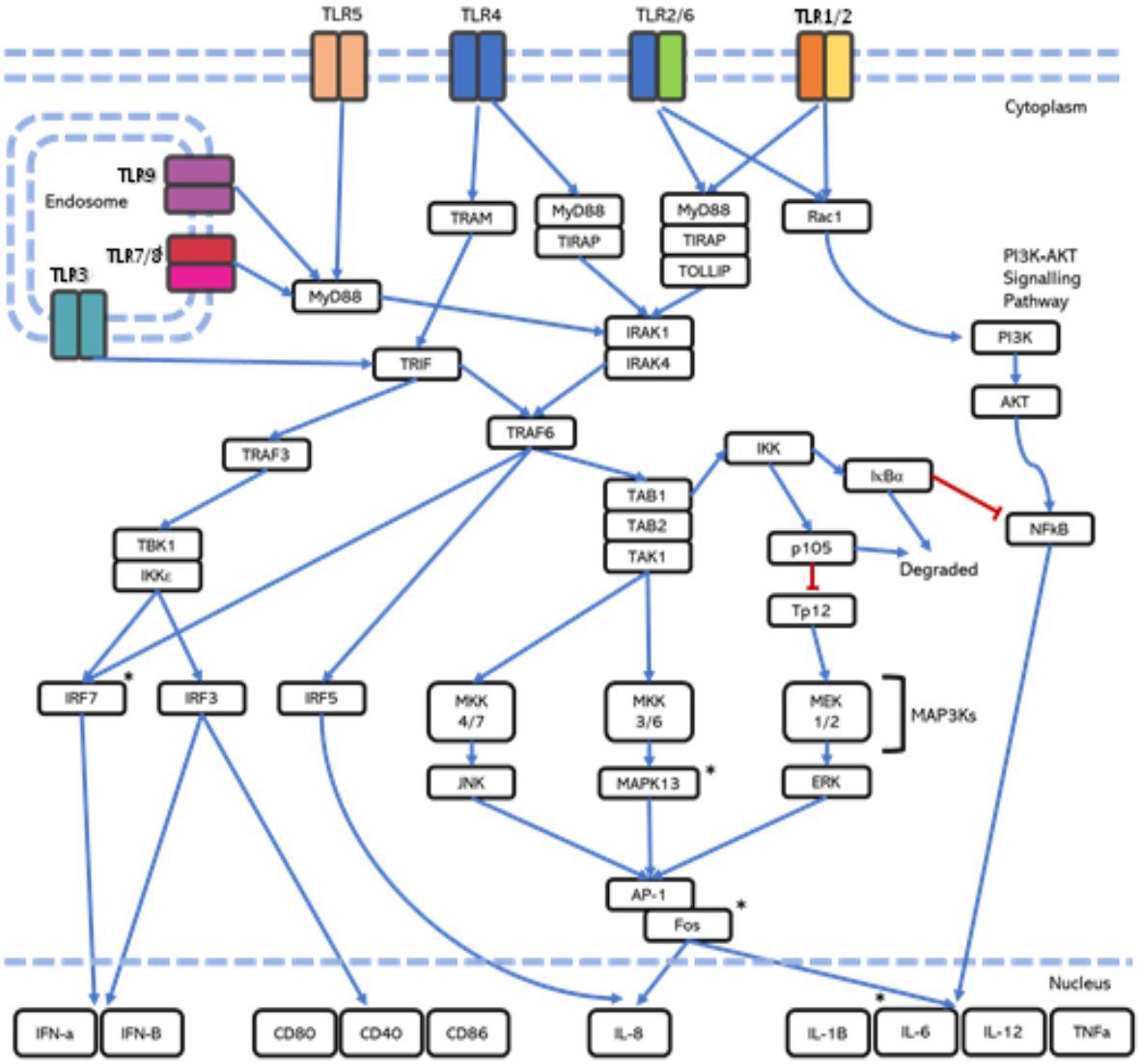



**Figure 1.10 Mammalian TLRs signalling pathway**

### 1.3.2 TLRs and salmonella

Salmonella spp. are highly infectious, clinically important and can range from self-limited gastrointestinal infections to toxic systemic infections (Li *et al.,* 2012).

*Salmonella enterica Typhimurium* is a Gram-negative bacterial pathogen that can infect a wide range of hosts, including humans and mice. *S. Typhimurium* has several PAMPs e.g. lipopolysaccharide (LPS), bacterial lipoproteins, flagellin, and CpG DNA and these are recognized by four TLR ligands TLR4, TLR2, TLR5 and TLR9 respectively (Zhang *et al.,* 2015).

The efficiency of detecting surface structures of *S. Typhimurium* can be enhanced by the variety and homo- and heterodimerization of TLRs (Takeuchi *et al.,* 1999). These include TLR2/6, TLR4, TLR5 and TLR9 (Rolls *et al.,* 2007). Each TLR recognizes a specific characteristic of the pathogen. For example, TLR4 is an essential receptor for pathogen-associated lipopolysaccharide (Fabrega and Vila, 2013) and activation of TLR4 is essential for the early innate immune detection of Gram-negative infections. This rapid detection by TLR4 has the potential as an immunotherapeutic strategy for detecting and eliminating specific bacterial infection (Leveque *et al.,* 2003). Another example of a *S. Typhimurium* PAMP is the protein flagellin which is specifically recognized by TLR5, and this is important for recognizing flagellated microorganisms. Flagellin binding to TLR5 recruits NF-kB pathway and induces synthesis of Type I cytokines, IFN-γ and IL-12, and a Th1 immune response (Tükel *et al.,* 2005).

Previous cohort studies have demonstrated that TLR4 is the main receptor for the bacterial lipopolysaccharide (LPS) (Liew *et al.,* 2005). LPS is a virulence factor in Gram-negative bacteria. Therefore, this TLR4 detection of LPS is important for controlling infection by pathogenic Gram-negative bacteria. LPS binding to TLR4 can induce the secretion of pro-inflammatory cytokines, nitric oxide, and other immune factors which can limit and resolve the infection (Leveque *et al.,* 2003).

The TLR5 ligands in *S. Typhimurium* include the salmonella-related flagella protein flagellin. This ligand is a specific component for TLR5 recognition of all flagellated bacteria (Hayashi *et al.,* 2001). TLR5 uses the NF-kB signalling pathway

to enhance the production of pro-inflammatory Type I cytokines, such as IFN-γ and IL-12, and can activate a Th1 adaptive response (Zhang *et al.,* 2014).

The Th1 response can contribute to curing the infection, an aberrant Th1 response can contribute to excessive inflammation and can cause tissue injury (Moss *et al.,* 2004). Thus, the TLR and Th1 responses have to be controlled and balanced carefully. This can be done by the production of other cytokines which can down-regulate the activation process. These anti-inflammatory cytokines e.g. IL-1Ra, IL-10, TGFß, are produced later in the gene expression and are involved in the resolution of inflammation after the infection recedes, and then in the repair process of immune-mediated tissue damage and restoration of homeostasis (Gewirtz *et al.,* 2001).

Generic anti-microbial responses following TLR signalling include activation of the transcription factor NF-κB and the production of reactive oxygen species (ROS). These factors in turn stimulate the expression of pro-inflammatory cytokines, enhance costimulatory molecules, and induce the expression of antimicrobial peptides including the inducible nitric oxide synthase (iNOs) and other defence molecules (Gowda, 2007).

Examples of the specific signalling pathways and functions of other TLRs include e.g. TLR2 activation which increases the permeability of epithelial tight junctions in the host intestine during microbial infection (Jung *et al.,* 2012).

The mechanisms behind this difference are poorly understood. Therefore, it is essential to understand the mechanism behind. As a core innate immune cell type, macrophage is a good model to investigate the response in infection disease. there is a growing literature describing new knowledge of phenotype, identity and function of macrophages differentiated in infected tissue from infiltrating inflammatory monocytes (Corliss *et al.,* 2016). We propose therefore to study the interaction of Salmonella and macrophages.

In this project we use macrophage as the basic cell type to analysis the function of IL-37 in Salmonella infection. There are some explanations about why we use macrophage as a research tool in infections. 1) Macrophages are parts of the initial defence towards pathogen infection, and are essential cell type in innate

immunity. Therefore, it provides unique possibilities to understand the contribution of this extremely dynamic cell subset in the reaction to infections (Torraca *et al.,* 2014). 2). During the infection, macrophage destroys bacteria by engulfing them in intracellular compartments, which they then acidify to kill or neutralize the bacteria. By counting alive bacteria in cell lysate, the invade bacteria number can be analysed. 3). Macrophages, especially murine RAW cell line, are widely used in many immune models, we also have human macrophage cell line THP-1 cell to compare the different gene expression and protein production under Salmonella infection.

## 1.4 Salmonella introduction

### 1.4.1 The characteristic of *Salmonella spp*.

Salmonella is a Gram-negative bacterium which belongs to the Enterobacteriaceae family. They have similar characteristics to the other family members of the Enterobacteriaceae; they are bacillus-shaped, non-spore-forming and facultatively anaerobic (Fabrega and Vila, 2013). *Salmonella spp.* is an enteral bacterium which can invade and attack the gastrointestinal tract of its host. Normally, they have a regular size of about 0.8–1.5 mm wide and 2-5 mm in length. Most strains of salmonella have peritrichous flagella which helps in the identification of *Salmonella spp.* (Fabrega and Vila, 2013). This assists their movement and motility, and can enhance their infectivity. There are more than 2000 serovars of *Salmonella spp.* reported up to 2011 (CDC, 2011) making this genus one of the most prevalent environmental pathogens. These pathogens can be categorized into two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* consists of approximately 2500 serovars which can be subdivided into 6 Salmonella subspecies. In *S. enterica subsp. enterica* (subspecies I), several members are associated with typhoid and paratyphoid infection-related disease. Due to the diversity of Salmonella serovars, they can infect a wide range of species, including humans and farm livestock (Percival *et al*, 2012).

Serovars of *S. enterica* can be classified according to the different disease they cause. *Salmonella enterica serovar Typhi* and Salmonella *enterica serovar Paratyphi* are the main pathogens which cause typhoid fever in human, which is also called S. *enterica serovar Typhi* or S. *Typhi* (Sabbagh *et al.,* 2010). However,

unlike the typical features of infection with other Gram-negative bacteria, the typhoidal Salmonella not only causes septic or neutrophilic shock but may lead to intestinal perforation and the potential to cause acute toxic encephalopathy (Tsolis *et al.*, 2008). In contrast, infection with other Salmonella serotypes e.g. *S. Typhimurium* is not associated with high mortality typhoid fever but can cause self-limiting diarrhoea, with approximately 10% of those infected developing a secondary bacteremia. These salmonella serovars are called non-typhoidal Salmonella (NTS). According to Sabbagh *et al.* (2010), these two groups of salmonella share approximately 90% structural homology and the 10% of structure variation contributes to virulence factors. Specific virulence factors can control their pathogenic potential e.g. LPS.

Of relevance to understanding these clinical difference, *Salmonella typhi* can infect human but not mice (Mittrücker *et al.*, 2000), thereby limiting laboratory research models, and the mechanism for this difference is not clear.

### 1.4.2 Molecular mechanism and immune response

The host immune response during *S. Typhimurium* infection consists of both B and T cell-mediated immunity and their associated mechanisms that regulate the protection reaction against infection (Mittrucker, 2002). Based on the research of Percival and Williams (2014), salmonella infection begins when sufficient numbers of pathogenic bacteria are ingested. Initially, these microbes form a site of infection and replication by adhering to the epithelia of the intestinal lumen. Once established, the salmonella can penetrate through the enteral epithelium. During this process, the invasive bacteria interfere with the function of the epithelial cell apical microvilli and damage the brush border to finish the penetration process. This process contributes to intestinal inflammation and diarrhoea. After penetration, the salmonella bacilli can enter the membrane-bound vacuole organelles in the cell cytoplasm where they can survive and proliferate (Hanna *et al.*, 2012). In this protective niche the bacilli produce toxic virulence factors which cause cell lysis allowing release of the pathogens from the intercellular environment and their dissemination. In addition, salmonella can breach the integrity of cell tight-junctions which can enhance the cytotoxic effects towards the host epithelial cells (Mittrucker, 2002). This pathogenic process decreases the host's ability to manage the normal dietary processes absorbing food and water.

In summary, salmonella can attach to and alter the permeability of the intestinal epithelial cell membrane resulting in cell lysis and diarrhoea (Percival and Williams, 2004).

The immune response to salmonella infection may attenuate the symptoms and eliminate the pathogens. Typically, an intact immune system can identify the salmonella antigens and initiate a protective immune response by activating Th1 lymphocytes. Several immune cytokines and anti-bacterial substances are generated by activated Th1 cell, such as IFN-γ and nitric oxide in order to assist the killing and removal of the salmonella (Moss *et al.,* 2004). When this is compromised, e.g. in patients with immunodeficiency or with an immature immune system, the immune response may be insufficient; for example, there may be insufficient production of the main therapeutic cytokine IFN-γ and may fail to recruit other parts of immune response allowing a much wider distribution of salmonella and clinical deterioration of the patient which can be life-threatening (Lee *et al.*, 2015). An additional hazard of salmonella infection includes septic shock due to the abundance of bacterial LPS and this is associated with increased mortality (Fierer and Guiney, 2001).

The Th1 immune response can usually eliminate this bacterial infection, however, when it becomes aberrantly over-activated it can contribute to sustained inflammation damaging host cells and cause pathology. Therefore, the appropriate degree of Th1 response should be governed carefully (Moss *et al.*, 2004). There are mechanisms which help to control an excessive inflammation. Anti-inflammatory cytokines such as IL-10 and TGF-β are released by Th2 cells, Treg cells, B cells, macrophages and DCs in order to decrease the immune-mediated damage to host cells, and to maintain a balanced response (Gewirtz *et al.,* 2001).

It has been reported that human and mouse also react differently in their immune response, especially in nitric oxide induction in *S. Typhimurium* infection (Vazquez-Torres *et al.,* 2008). From *in vitro* result, human macrophages are less sensitive than murine in the infectious immune response, and have comparably lower cytokine production and anti-bacterial factor production than mice, e.g. nitric oxide (*ibid*). Due to the features of *S. Typhimurium,* the invaded bacteria can lead to a conserved fimbrial gene cluster in mice, which is a main macrophage

stimulating factor. However, similar situation cannot be observed in human sample. Moreover, the reason why human have significantly lower nitric oxide production than mice is not clear. This evidence indicates that the inflammation responses may have variations in different species (Tükel *et al.*, 2005).

### 1.4.3 Salmonellosis

Salmonellosis is the term for a disease caused by Salmonella infection. Infection leads to many manifestations of disease: enteric fever (typhoid fever), enterocolitis and systemic illness (Trebichavský and Šplíchalová, 2010). Among these diseases, typhoid fever and enterocolitis represent a severe social health concern worldwide, especially in developing countries such as Lebanon and India (Malaeb *et al.*, 2016). Without efficient clinical treatment, salmonellosis leads to high morbidity and mortality. This can also be the case in developed countries that have well-established and organized healthcare systems. For example, in America, approximately 10% of foodborne diseases are caused by nontyphoidal *Salmonella spp*. (Scallan *et al.*, 2011), and these diseases are a considerable individual, social, clinical and economic burden. In 2011, gastrointestinal infections caused by salmonella resulted in more than 1 million cases, resulting in thousands of hospitalizations and nearly 600 deaths in America (Scallan *et al.*, 2011). The associated costs were approximately 16 billion dollars annually for treating foodborne disease; predominantly associated with salmonella infection. Furthermore, several serotypes may cause similar symptoms in human and animals which contribute to the prevalence of salmonella infection. Therefore, *Salmonella spp.* is one of the most severe and prevalent pathogens during the last century.

According to Coburn *et al.* (2007), the annual morbidity of Salmonellosis in humans was approximately 1.3 billion worldwide. Moreover, the global mortality associated with non-typhoid Salmonella serovars is estimated at approximately 3 million per year. Young adults (20-39 years of age) were the most susceptible to Salmonella infection (25% of cases). Children and teenagers were less susceptible and the elderly and newborns were the least susceptible to Salmonella infection (Malaeb *et al.*, 2016). Among these groups those with a compromised immune system are most susceptible. More than 60% of salmonellosis occurs during the summer months. Therefore, an increased rate of Salmonella infection is

associated with environmental and public health situations; one main cause is contamination during food processing, storage and transportation (Bajpai *et al,* 2012).

When individuals come into contact with foodstuff that is contaminated with Salmonella, they will become potential hosts of the bacteria (Malaeb *et al.,* 2016). The pathogen can adhere to the intestinal mucosa to initiate site of infection (de Jong *et al.*, 2012). Typical symptoms include fever, diarrhoea and abdominal cramps. For diagnosis, a positive foecal culture result is be required to confirm an infection. Usually these symptoms are self-limiting and recovery after 2-5 days. In some case the infection can develop from mild to severe, with the potential to become life-threating (Percival *et al.,* 2012). Those patients infected with salmonella who develop bacteremia and systemic infection have a much greater morbidity, mortality and treatment failure.

### 1.4.4 Treatment

The most common treatment for salmonella infection is antibiotic therapy. There is clear evidence of antibiotics in treating S. *Typhi* infection, with a successful result in managing the process of typhoid fever (Onwuezobe *et al.,* 2012). However, some nontyphoidal *Salmonella spp.* do not respond to antibiotics well. The misuse of antibiotics may cause clinical burden to the patients.

The general overuse of antibiotics worldwide has resulted in the emergence of Salmonella strains that are resistant to many of antibiotics, and this is of considerable clinical concern worldwide (Hanna *et al.*, 2012). Moreover, the increasing rate of foodborne infections also contributes to the environmental spread of resistant Salmonella species. These resistant bacteria can survive extreme environments and strong antibiotics. Based on the research by CDC (2011), 70% of foodborne disease pathogens are resistant to at least one antibiotic. According to Cosby *et al.* (2015), there are two mechanisms to explain the increasing resistance rate: 1. enzymatic inactivation of the antibiotic by chloramphenicol O-acetyl-transferase, and 2. removal of the antibiotic by an efflux pump. Therefore, it is crucial to find additional strategies or treatments which can control infections by Salmonella, especially nontyphoidal salmonella *spp.*

## 1.5 Project aim and hypothesis

Till date, IL-37 relate studies are mainly focused on IL-37b isoform and demonstrate that IL-37b is an important immunosuppressive cytokine in TLR response in inflammatory diseases. However, the function and importance of other isoforms, especially the isoform a which own a unique exon 3, is still unknown. As IL-37a carries the same receptor-binding domain as IL-37b, the two isoforms may share the same receptor and signalling via similar pathway in the regulation of TLR function. However, the unique N-terminal domains may confer IL-37a additional function in nuclear translocation and gene regulation. Therefore, it is important to compare the functional difference between these two isoforms and find out the mechanism behind. My hypothesis are: 1)Based on its unique structure, IL-37a may be a novel immune suppressive cytokine and a nuclear factor, thereby IL-37a may be more effective than the well-studied IL-37 isoform-IL-37b. 2)Given its immunosuppressive function, IL- 37a may promote Salmonella infection in macrophages *in vitro* by inhibiting immune response *in vivo*. 3) IL-37 may also reduce Salmonella- mediated pathology by inhibiting pro- inflammatory cytokine productions *in vivo*.

In this thesis, I aim to produce IL-37a and IL-37b recombinant proteins and transgenic mice as a research tool for comparing the functional difference between these two isoforms. Moreover, I will investigate the regulatory effect of IL-37a on TLR response in vitro and in the infectious disease model, using salmonella. Furthermore, I also explore the underlying molecular mechanism by which IL-37a and IL-37b differently affect TLR4 response in immune cells using transcriptomic analysis. All these results are focused on addressing the main issue: What is the difference between IL-37a and b in TLR stimulations and by what mechanism.

# Chapter 2

# Materials and methods

# 2. Material and method

## 2.1 Mice

All mice (wildtype/transgenic mice) were housed in the Biological Services facility, University of Glasgow, following the United Kingdom Home Office regulation. All the animal procedures were undertaken at the Central Research Facility (CRF) of the University of Glasgow under project license NO. PD13F11FC and 60/4405; the researcher also hold a personal license (NO. IFB9F1C16). CRF stuff have specialized facilities for animal housing and expertise for animal handling and experiment. Mice for experiment were typically 6-8 weeks old. Mice strains are summarized in Table 2.1. All transgenic mice were randomly distributed into several groups in similar number and identified by genotyping.

All mice had clean food and water without limitation; and for the purpose of environmental enrichment their cages were supplied with bedding and other support materials when necessary. For individual assessment, all mice were ear marked. The maximum capacity of each cage was five (marked as no earmarked, left earmarked, right earmarked, double earmarked and two left earmarked).

| strain | genetic modification | source |
|---|---|---|
| Balb/C | wild-type | Charles River lab |
| C67BL/6 | wild-type | Charles River lab |
| C67BL/6 | IL-37a transgenic (TgIL-37a) | Bred at University of Glasgow |
| C67BL/6 | SIGIRR gene knockout (SIGIRR KO) | Bred at University of Glasgow |
| C67BL/6 | IL-37a transgenic with receptor knockout (TKIL-37a) | Bred at University of Glasgow |

**Table 2.1 Mice strain used in this project**

## 2.2 Cell stimulators

Cell stimulators, working concentrations and suppliers are summarised in Table 2.2.

| cell stimuli | Supplier | working concentrations |
|---|---|---|
| PAM3 | Thermo Fisher | 10ng/ml |
| FlAst | Thermo Fisher | 10ng/ml |
| TPA/PMA | Sigma-Aldrich | 50ng/well (24 well plate) |
| Lipofectamine™ RNAiMAX | Thermo Fisher | 25ul/ well (96 well plate) |
| IMQ | InvivoGen | 5ug/ml |
| CpG | InvivoGen | 3ug/ml |
| LPS | Thermo Fisher | 100 ng/ml, 10ug/ml |
| rhIL-37a | House made | 50 ng/ ml |
| rhIL-37a (mutant monomer) | House made | 50 ng/ ml |
| rhIL-37b | House made | 50 ng/ ml |
| rhIL-37b (mutant monomer) | House made | 50 ng/ ml |

**Table 2.2 Stimulators used in experiments**

## 2.3 Antibodies

Antibodies used for ELISA and western blot used are summarized in Table 2-3.

| Antibody | Produced in | Stock concentration | Dilution | Company |
|---|---|---|---|---|
| Anti-6 His tag | Mouse | 0.1 mg | 1 :1000 | Abcam |
| horseradish peroxidase (HRP) | Rabbit | 1.0 mg | 1:10000 | Abcam |
| Biotinylated anti-human IgG | Mouse | 0.5 mg/ml | 1 : 500 | BD pharmingen |
| Biotinylated anti-mouse IgG | human | 0.5 mg/ml | 1 : 500 | BD pharmingen |

**Table 2.3 Antibodies used in ELISA and western blot**

## 2.4 Chemicals and reagents

Chemicals and buffers used are listed in Tables 2-4 and 2-5.

| Chemicals | Supplier | Usage |
|---|---|---|
| 10% Ammonium persulfate (APS) | Sigma-Aldrich | Protein purification |
| 10% SDS | Sigma-Aldrich | Protein purification |
| 1M /1N Acedic acid | Sigma-Aldrich | ELISA |
| 1M /1N hydrochloric acid | Sigma-Aldrich | ELISA |
| 3,3',5,5'-Tetramethylbenzidine (TMB) | eBioscience | ELISA |
| 30% Acrylamide | Sigma-Aldrich | Protein purification |
| Agarose | Sigma-Aldrich | Genotyping |
| Ampicillin | Sigma-Aldrich | Protein purification |
| DNAseI | Sigma-Aldrich | Protein purification |
| EDTA-free complete protease inhibitor cocktail | Sigma-Aldrich | Protein purification |
| Ethidium Bromide | Invitrogen | Genotyping |
| Imidazole | Sigma-Aldrich | Protein purification |
| Isopropyl ß-D-1-thiogalactopyranoside (IPTG) | Sigma-Aldrich | Protein purification |
| Kanamycin | Sigma-Aldrich | Protein purification |
| Lysozyme | Sigma-Aldrich | Protein purification |
| N,N,N′,N′-tetramethylethylene-diamine (TEMED) | Sigma-Aldrich | Protein purification |
| Protease Inhibitor Cocktail | Sigma-Aldrich | Protein extraction |
| Tris Acetate-EDTA (TAE) | Sigma-Aldrich | Genotyping |
| Tween® 20 | Sigma-Aldrich | ELISA |

**Table 2.4 Chemicals used throughout the project**

| Chemicals | Supplier | Usage |
|---|---|---|
| 10% Ammonium persulfate (APS) | Sigma-Aldrich | Protein purification |
| 10% SDS | Sigma-Aldrich | Protein purification |
| 1M /1N Acedic acid | Sigma-Aldrich | ELISA |
| 1M /1N hydrochloric acid | Sigma-Aldrich | ELISA |
| 3,3',5,5'-Tetramethylbenzidine (TMB) | eBioscience | ELISA |
| 30% Acrylamide | Sigma-Aldrich | Protein purification |
| Agarose | Sigma-Aldrich | Genotyping |
| Ampicillin | Sigma-Aldrich | Protein purification |
| DNAseI | Sigma-Aldrich | Protein purification |
| EDTA-free complete protease inhibitor cocktail | Sigma-Aldrich | Protein purification |
| Ethidium Bromide | Invitrogen | Genotyping |
| Imidazole | Sigma-Aldrich | Protein purification |
| Isopropyl ß-D-1-thiogalactopyranoside (IPTG) | Sigma-Aldrich | Protein purification |
| Kanamycin | Sigma-Aldrich | Protein purification |
| Lysozyme | Sigma-Aldrich | Protein purification |
| N,N,N′,N′-tetramethylethylene-diamine (TEMED) | Sigma-Aldrich | Protein purification |
| Protease Inhibitor Cocktail | Sigma-Aldrich | Protein extraction |
| Tris Acetate-EDTA (TAE) | Sigma-Aldrich | Genotyping |
| Tween® 20 | Sigma-Aldrich | ELISA |

**Table 2.5 Various buffers and their composition used throughout the project**

## 2.5 Tissue cell and cell-line culture

Primary cells and cell lines were cultured in complete medium (RPMI 1640 or DMEM, 10% heat-inactivated FBS, 100U/ml Penicillin, 100µg/ml Streptomycin, 2 mM L-glutamine; Thermo fisher, UK), unless otherwise specified. Cell cultures were maintained in a humidified chamber (Heraeus Instruments, Germany) supplemented with 5% $CO_2$ at 37.0°C. Viability of the cells was evaluated by trypan-blue staining [0.1% (w/v) Trypan Blue (Sigma-Aldrich, USA)] and counted using Neubauer haemocytometers (Weber Scientific International Ltd, UK) on a Nikon Labphot microscope.

### 2.5.1 Primary culture of murine splenocytes

Mice were sacrificed by exposing to $CO_2$. The animals were then placed on a clean dissection board and rinsed with 70% reagent alcohol. The spleens were harvested by incision of the abdominal cavity then put in ice-cold complete RPMI 1640 (Roswell Park Memorial Institute – 1640, Gibco®) culture medium for short storage. The splenocytes were isolated using 100um sterile EASYstrainer™ (Greiner bio-one, UK) which place on cell culture petri dish (SIGMA). Cells were gently separated from the tissue by 2ml syringe smashing. All cells were collected in 50ml falcon tube with RPMI media, and then washed with PBS 1X. Red blood cells were lysed with appropriate lysis buffer (SIGMA) following manufacturer's instructions. Upon to times washing with RPMI, the cells were placed and cultured in complete RPMI media in appropriate concentration.

### 2.5.2 Peritoneal macrophage preparation

To prepare peritoneal macrophages, WT and IL-37a transgenic mice were separated into two groups (a least 3 mice per group) before injection. 50ml 5mM sodium m-periodate (Sigma-Aldrich) buffer was prepared for the following macrophage stimulation and kept on ice. 1ml of the buffer was drawn into the right part of peritoneal cavity of each mouse by intraperitoneal injection. Mice were kept in the original cages for another 4 days till cell harvest. After culled in a $CO_2$ chamber following the ethics statements, mice peritoneal cavity was washed by 5ml of ice-cold PBS buffer and the peritoneal macrophages were

collected with PBS buffer using a syringe. The peritoneal macrophages were incubated in complete RPMI culture medium till further experiment. They cells became adhere in 6 hours after collection.

### 2.5.3 Human peripheral blood mononuclear cell (PBMC) isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were separated from buffy coat of healthy donors by density gradient centrifugation using Ficoll. In more details, whole blood was diluted with PBS 1:1, and slowly added on the top of 3ml Ficoll-1077 (SIGMA) previously added in a 15ml falcon tube. The sample was centrifuged at 400g at room temperature for 30 minutes. The whole blood was separated into different layers by ficoll-1077 after centrifugation. Serum in the upper layer and the PBMC cell ring in the mid layer were collected by plastic pipette. Then the PBMCs were washed twice by sterile PBS and cultured in complete RPMI for further use.

### 2.5.4 THP-1 cell culture

#### 2.5.4.1 THP-1 monocyte culture

Human monocyte THP-1 cells were cultured in complete RPMI 1640 medium in a humidified incubator (Heraeus Instruments, Germany) at 37°C and 5% $CO_2$.

#### 2.5.4.2 THP-1 Cell Differentiation

THP-1 monocytes were collected with culture media and centrifuged at 1200rpm for 5 minutes. After discarded the supernatant, monocytes were then resuspended in 4ml fresh complete RPMI for cell counting. Suspension monocytes were counted with 0.1% (w/v) Trypan Blue (Sigma-Aldrich) stain in Neubauer haemocytometer and check under a Nikon Labophot microscope (x10 magnification). Monocytes were then placed at a density of $1.2x10^5$ cells/ml per well into 24-well cell culture plate and incubated for 24 hours under 37°C, 5% $CO_2$ culture condition. After 24 hours' incubation, cell density was calculated again with same method with additional 50ng of Tetradecanoyl phorbol acetate/ *phorbol 12-myristate 13-acetate* (TPA/PMA) (Sigma-Aldrich) to each well for 48 hours to differentiate into adherent macrophage-like cells. Before infection process, the morphology and adhesion condition of THP-1 cells was observed in order to confirm the efficiency

of macrophage differentiation. The differentiation efficiency was around 95% for all the samples. Moreover, the cells were washed twice with RPMI 1640 media with 3% FCS and 2mM L-glutamine. Fresh culture medium was applied after the old medium was discarded in order to remove the undifferentiated monocytes and supply a better experiment condition for salmonella infection.

### 2.5.5 Murine macrophage (RAW 264.7) cell culture

RAW 264.7 cells (murine macrophages) were also used in this project for comparing the infection result between human and murine macrophages. Raw cells (generation 7) were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco®) media with 10% FCS, 2mM L-glutamine and 1% penicillin–streptomycin. Owing to the density of THP-1 cells, 2.8x10^5 RAW cells were placed in 24-well cell culture plates and media was replaced by complete RPMI1640 media 24h before infection. Therefore, cell density of THP-1 macrophage and RAW cells for infection would be similar. After that, old cell culture media was discarded and replaced by RPMI 1640 media with 10% heat inactivated FBS in addition to 2mM L-glutamine in 24h. The purpose of this step is to make the RAW cell more sensitive to the infection as well as maintain in similar culture condition as human macrophage. Same as THP-1 cell, the cell was washed twice with RPMI 1640 media with 3% FCS and 2mM L-glutamine just prior to the bacterial infection.

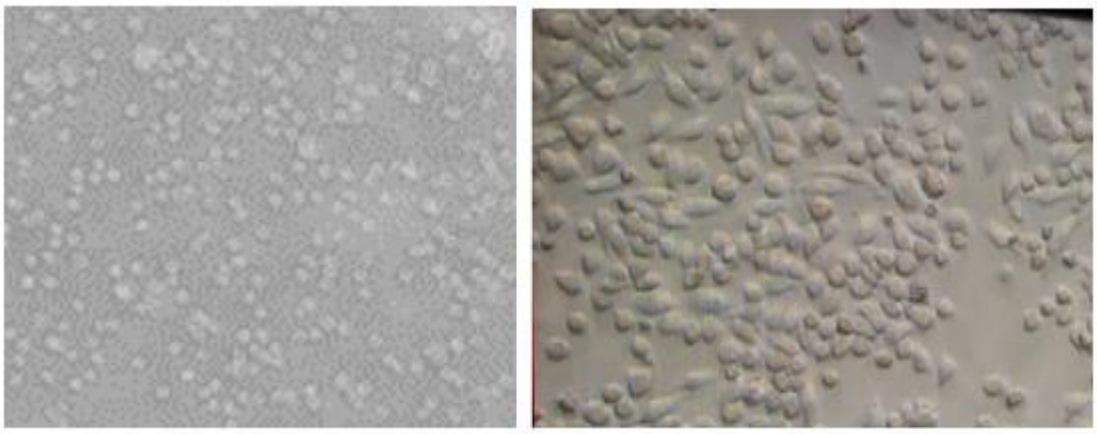


**Figure 2.1 Comparison of THP-1 monocytes and macrophage (Magnification: x10)**

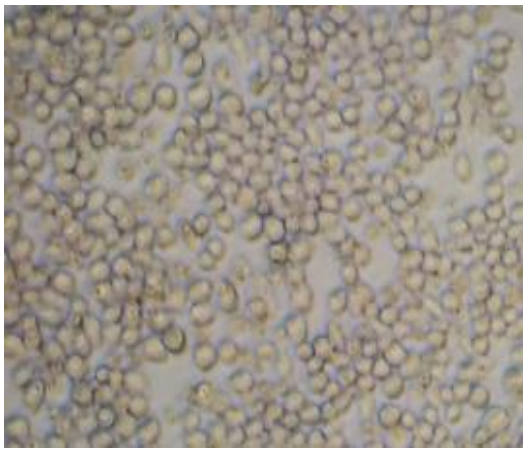


**Figure 2.2 Murine RAW macrophage (Magnification: x10)**

## 2.6 Recombinant IL-37a and b protein production

### 2.6.1 Recombinant IL-37 a and b protein expression

#### 2.6.1.1 Production of competent cells and transformation of *Escherichia coli (E. coli)*

*E. coli* host strain BL21 (DE3) was first incubated in 10ml LB broth in a shaker at 37°C overnight. After incubation, the wild strain was then cultured on LB agarose plate with chloramphenicol antibiotic to avoid contamination. The stock can be stored for further process at -80°C.

In order to prepare competent *E. coli*, 500µl of host cells after overnight culture were added in a conical flask with 50ml LB broth. Then the culture flask was placed in a shaking incubator at 37°C, culture for 3-4 hours. After cell culture, cells were able to reach the optimal optical density (OD). Required OD of the bacteria culture is around 0.3-0.4 under 600nm wavelength measurement. Cells were then centrifuged at 3000rpm for 10 minutes. After discarding the supernatant, cell pellets were re-suspended in ice cold calcium chloride ($CaCl_2$) buffer (0.1M). Because the cell become weak after centrifuge, 1ml of $CaCl_2$ buffer was added and mixed gently; then volume was made up to 25 ml (half the original volume of LB media). As the $CaCl_2$ in the room temperature is toxic for the cells, cells were incubated on ice for at least 1 hour. Moreover, cells were centrifuged again at 2500rpm for 5 minutes. After adding another 2ml of 0.1M $CaCl_2$, cells became competent and ready to be used as a host bacterium for transformation.

For transformation, 200µl of competent bacteria were separated in two groups in 1.5ml sterile Eppendorf tubes labelled as test and control samples. 1ng of plasmid constructs which contains specific gene encoding protein (IL-37a and IL-37b) was added to the tubes of the test group. Tubes of both groups were place on ice and incubated for 45 minutes, then placed in water bath (42°C) for 90 seconds to open the cell membrane and assist plasmid insertion into the cell. After heat shock, tubes were immediately placed on ice again. Additional 800µl of LB broth was added into each tube and bacteria were incubated at 37°C for 30 min, the tubes were gently inverted every 5 minutes to avoid cells destruction.

### 2.6.1.2 Plasmid DNA extraction

Plasmid DNA was extracted from *E. coli strain* DH5α using the QIAprep®Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

### 2.6.1.3 Storage and bacterial growth conditions

All bacteria stains were stored in 50% glycerol stocks at -80ºC condition. To recover the frozen bacteria, the stock was thawed in a 37 ºC water bath and placed on a LB agar plate for forming colony. Single colony was then transferred into 1ml LB broth culture media with 10% glycerol. After transformation, *E. coli* strains were routinely grown in typical LB agar or broth in the stable 37ºC incubator. The selective culture media contained antibiotics which related to the transferred plasmid. We used Ampicillin or Kanamycin (100µl/ml) for IL-37a and b respectively.

### 2.6.1.4 Expression of recombinant IL-37a and IL-37b

The recombinant IL-37 proteins (IL-37a and b) were produced in *E. coli* BL21 (DE3). Transformed DNA can be specially expressed with IPTG induction. To optimize the expression condition, the researcher tested variant induction conditions and the successfully expressed recombinant IL-37a and b in large scale.

### 2.6.1.5 Recombinant IL-37a and b test expression

*E. coli* BL21 (DE3) used as a template to express the IL-37 protein by transformed with the pET-28a vector for IL-37 (IL-37a and IL-37b). The transformation step had done for both pET-28a vector for IL-37a and IL-37b respectively. After the transformation, the successfully transformed *E. coli* were selected by specific antibiotic bacteria culture LB agarose plate and a single colony was picked up by tips and growth in Luria Bertani (LB) medium supplemented with 100µg/ml Ampicillin/ kanamycin at 37°C overnight. Moreover, the bacteria culture mixture was diluted in fresh LB medium (1:50 dilution) which containing kanamycin and grown at 37°C under continuous shaking (180rpm, 37°C), until the absorbance (OD level) at 600nm reached 0.6. IL-37 expression was induced by adding 1 mM IPTG (SIGMA, UK) to the successfully transformed *E. coli* BL21 (DE3), and the bacteria were incubated at 37°C for several time points (1, 2, 3, 4, 5, 6, and 24 h), the

culture mixture were collected at each time point respectively and stored in -20°C. The expression level was evaluated by 15% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). In order to test at which time points the IL-37 protein has the best expression level and give a proof to optimize the culture conditions.

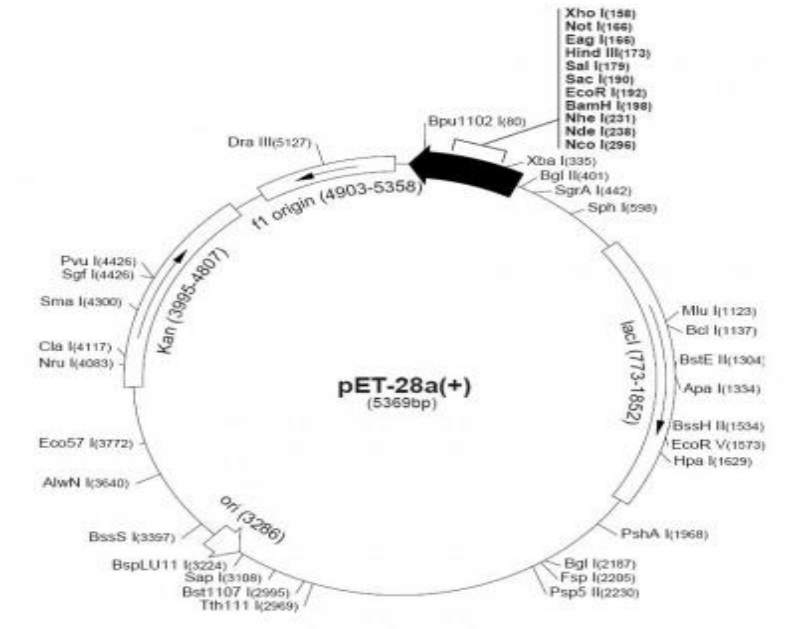



**Figure 2.3 the construction of IL-37**

#### 2.6.1.6 Large scale expression of recombinant IL-37a and b in *E. coli*

One single colony of *E. coli* which had been successfully transformed with IL-37a or b DNA (pET-28a/IL-37b or pET21 a/IL-37a) was picked up from selected plate and grown at 37°C in Luria Bertani (LB) medium supplemented with 100μg/ml Ampicillin/ kanamycin overnight. The mixture of *E. coli* culture was diluted 100 times in fresh LB medium 6.25 ml for a 625ml culture) which containing Ampicillin/ kanamycin (100ug/ml) and grown at 37°C in a shaking (180rpm, 37°C) incubator, until the OD at 600 nm reached 0.6. After that, IL-37 expression was induced by adding 1 mM IPTG to the successfully transformed *E. coli* BL21 (DE3), and the bacteria were incubated at 37°C for 4 hours. After the culture medium was cooled enough, the bacteria were harvested by 4°C centrifugation at 5000 rpm for 15 minutes using the Avanti J-26PI centrifuge with a JA-25.50 fixed angle rotor (Beckman Coulter, Inc.). Furthermore, the culture Supernatants were decanted, and the pellet was stored at −80°C overnight.

### 2.6.2 recombinant IL-37a and IL-37b protein purification

The recombinant IL-37a and b with 6His-tag can be purified using immobilized metal affinity chromatography (IMAC) method by Ni-NTA column. Dialysis and gel filtration, the purity of recombinant protein was further checked by SDS-PAGE gel and confirmed by western blot in Dr Xu's lab. Protein concentrations were measured by Coomassie Blue assay with endotoxin removal. The recombinant proteins are ready for cell assay after purification.

#### 2.6.2.1 Bacteria cell lysate Preparation

The next day, the transformed bacteria pellet was collected from -80°C and resuspended in 35 ml of bacteria lysis buffer then vortexed till the pellet was completely dissolved. Furthermore, the lysate was sonicated on ice for further disruption (15 second sonication on, 45 seconds off for 10 cycles) until the solution was no longer viscous. After sonication, an 18000rpm centrifuge was performed for removing the insoluble substances at 4°C for 30 min. The supernatant was collected and kept on ice for protein purification.

### 2.6.2.2 Purification of recombinant IL-37a and IL-37b in Ni-NTA column

The Ni-NTA column (GH Healthcare) was used for IL-37a and IL-37b protein purification. The column was equilibrating with binding buffer (500 mM NaCl, 20 mM tris base, 10 mM imidazole, PH= 7.5 filtered by Büchner funnel) and elusion buffer (500 mM NaCl, 20 mM tris base, 10 mM imidazole, PH=7.5 filtered by Büchner funnel). The bacteria supernatant was loaded into the column after centrifuge and the whole process was controlled by the column machine and monitored by the Ni-NTA column system (Primeview). In the column, the proteins of loading mixture were binding to the column then eluted with the elution buffer. After checking the protein peak of the Ni-NTA column system on the screen, several tubes were selected, and samples were picked from them to prepare for SDS–PAGE analysis process.

### 2.6.2.3 Sodium dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

Purified IL-37 proteins (IL-37a/b) were treated with sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. A resolving gel and stacking gel were prepared before the experiment using the constituents in the Table 2.6a and b. The recombinant bacteria extract or the purified proteins were resuspended in SDS–PAGE loading buffer with 95°C heating on a dry heat block for 5-10 minutes. Then the samples were centrifuged at 12000rpm for 2 minutes and 10µl samples were loaded per well on a 15% SDS–PAGE for 120volt, 40 minutes' electrophoresis in the electrophorese tank (Sigma, UK). After electrophoresis, the denatured proteins were stained with 0.1% Coomassie Brilliant Blue R-250 solution for 1 hour to become visible and de-stained by gel de-stain buffer overnight.

Constituents of resolving gel for SDS-PAGE

| Reagent | Amount |
|---|---|
| Distilled water (D.H2O) | 4.4 ml |
| 1.5M Tris PH=8.8 | 5.2 ml |
| 30% acrylamide mixture | 10 ml |
| 10% sodium dodecyl sulphate (SDS) | 200 µl |
| 10% ammonium per sulphate (APS) | 200 µl |
| tetramethyl ethylenediamine (TEMED) | 8 µl |
| Total amount (for 4 gels) | 10 ml |

**Table 2.6a Constituents of resolving gel for SDS-PAGE**

Constituents of stacking gel for SDS-PAGE

| Reagent | Amount |
|---|---|
| Distilled water (D.H2O) | 5.5 ml |
| 1M Tris PH=6.8 | 1 ml |
| 30% acrylamide mixture | 1.3 ml |
| 10% SDS | 80 µl |
| 10% APS | 80 µl |
| TEMED | 8 µl |
| Total amount (for 4 gels) | 8 ml |

**Table 2.6b Constituents of resolving gel for SDS-PAGE**

#### 2.6.2.4 Dialysis condition

IL-37 proteins were collected following the result of SDS-PAGE gel and dialyzed in dialysis buffer for further purification with gel filtration using S75 column. 5 litres of plastic breakers were used for protein dialysis, the proteins (IL-37a and IL-37b) after NI-NTA column purification were put into a dialysis tubing cellulose membrane (Sigma) in the dialysis buffer with a magnetic stirrer at 4°C overnight. The dialyzed sample can be used for further purification such as gel filtration after centrifuge to remove the possible denatured protein precipitation. After the gel filtration, proteins were dialyzed again in PBS buffer. The dialyzed proteins kept at -20°C for protein bioassay.

#### 2.6.2.5 Gel filtration

Gel filtration was further used to separate the protein of interest by their size. After purification with NI-NTA column and checking protein purity by Western Blot, the IL-37 protein was further purified by gel filtration chromatography. HiLoad Superdex 200 PG column (30 × 10 mm, Pharmacia GE Health Sciences) was used to purify the protein mixture after Ni-NTA column was confirmed by SDS-PAGE gel. Before the mixture loading, the column was equilibrated for 5 hours with 50 mM acetate buffer which contained 100mM KCl (pH =5). The mixture was loaded with the assist of 10ml syringe, all the liquid was applied on the column and then elute with the elution buffer (200mM NaCl and 50mM Tris) at a flow rate of 0.33ml/min. Fraction was automatically collected after draining 80% of the void volume by the Fraction Collector Frac-920 obtained from Pharmacia GE Health Sciences. Each collection tube contained 2ml of eluted protein with elution buffer. Different protein can be separated in this step with the different peak which analysis by system. The concentration of the protein fractions was detected by photometer at 280nm OD level.

#### 2.6.2.6 Western blot

Western blotting technique was used for testing the protein conformation and specificity of IL-37 (IL-37a and IL-37b). The purified protein was collected and separated by 20% SDS–PAGE gel. Then samples were transferred into a polyvinylidene difluoride (PVDF) membrane (Millipore), followed by blocking for

unspecific antibody binding with 5% defatted milk. The membrane was washed with PBS buffer with additional 0.05% Tween20 3 times and incubated with the diluted primary antibodies (human anti-His6 tag monoclonal antibody, ABD) in 1:500 dilution at 4°C overnight. Furthermore, the HRP-conjugated rabbit anti-mouse (Abcam) was used as a secondary antibody, incubated with the samples at room temperature for 2 hours. After buffer and antibody preparation, the western papers were soaking in cold buffer with sponges. The transfer process under 100V, constant 350mA for 1 hour using the same electrophorese tank as for SDS-PAGE gel (Sigma, UK). The result of Western blot was detected by luminal system (Thermo Fisher scientific).

#### 2.6.2.7 Protein concentration measurement

IL-37 protein concentration was measured with Coomassie Blue Protein Assay Kit (Thermo Scientific). Briefly, 2-fold diluted protein standard has been prepared (Bovine serum albumin standards; Thermo Scientific, USA), Add 50µl of sample and standard into Eppendorf tubes with additional 1.25ml of Coomassie reagent into each tube; use PBS as a blank. Mix the sample and standard well and incubate at room temperature for 10 minutes. Mix well then transfer 150µl of samples and standards into each microplate wells; Measure the absorbance of supernatants at 465nm. The standard curve of Coomassie Blue essay was developed based on absorbance and concentration (µg/ml) of the protein standards. Protein concentration of the IL-37 was calculated by reference to the standard curve.

#### 2.6.2.8 Endotoxin removal

Endotoxin which contained in the purified recombinant IL-37a and IL-37b protein products was removed by the Package Insert EndoTrap endotoxin removal column (EndoTrap HD). The purification was following the manufacturer`s manual. To be more specific, the column was placed on a shelf then loose the cap on the top and remove the cap at bottom of the column. The liquid in the column can drain from the column with gravity. After all the liquid run out from column, the column was then activated with 3ml regeneration buffer and wash the column twice. After that, fill the column with the recombinant IL-37 sample after NI-NTA column purification. The flow through of the column was collect directly and store on ice. The column can be reuse for several experiment with the regeneration buffer.

### 2.6.2.9 Endotoxin detection

The endotoxin level in the recombinant IL-37 was measured by Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation kit (Thermo Fisher Scientific, USA). The test process was performed following the manufacturer`s instructions: Firstly, IL-37 protein PH level was adjusted to 6-8 using endotoxin-free 0.1M NaOH or HCL solution. The microplate was heated before experiment on a heating block (Thermo Fisher Scientific, USA) at 37°C for 10 minutes. Dispense 50µl of standard dilutions and samples replicate to appropriate wells carefully in order to keep an adding manner and add same amount of LAL reagent water into empty wells as blank. Moreover, additional 50µl of LAL reagent was added into each well by pipette, keep the same order as the sample added in. Shaking the plate gently on a plate orbital shaker (AROS) for 10 seconds to mix the sample with reagent and incubate at 37°C for another 10 minutes. Add 100µl substrate solution into the whole plate and cover the plate by a plate sealer, incubate at 37°C for 6 minutes. Stop the reaction by adding 50µl of stop solution in the same manner as adding samples. Read the plate with the 96 wells plate reader (Dynex technologies) at 506-410nm wavelength. Measure the endotoxin level by optical density (OD) of standards and samples which based on the Endotoxin standard curve.

### 2.6.2.10 native gel preparation

The native and mutant IL-37 proteins (monomer and dimer IL-37a and b) were confirmed by native gel in un-denature condition. A resolving gel and stacking gel were prepared before the experiment which using the constituents in the Table 2.7a and b. The recombinant bacteria extract or the purified proteins were resuspended in native loading buffer 1:10. Then the samples were centrifuged at 12000rpm for 2 minutes and loaded at 10µl samples per well on a 15% native gel for 120V, 40 minutes' electrophoresis in the electrophorese tank (Sigma, UK) on ice with native gel running buffer. After electrophoresis, all the proteins were stained with 0.1% Coomassie Brilliant Blue R-250 solution for 1 hour to become visible and de-stained by gel de-stain buffer overnight.

Constituents of resolving gel for native gel

| Reagent | Amount |
|---|---|
| Distilled water (D.H2O) | 4.4 ml |
| 1.5M Tris PH=8.8 | 5.2 ml |
| 30% acrylamide mixture | 10 ml |
| 10% ammonium per sulphate (APS) | 200 µl |
| tetramethyl ethylenediamine (TEMED) | 8 µl |
| Total amount (for 4 gels) | 10 ml |

**Table 2.7a Constituents of resolving gel for native gel**

Constituents of stacking gel for native gel

| Reagent | Amount |
|---|---|
| Distilled water (D.H2O) | 5.5 ml |
| 1M Tris PH=6.8 | 1 ml |
| 30% acrylamide mixture | 1.3 ml |
| 10% APS | 80 µl |
| TEMED | 8 µl |
| Total amount (for 4 gels) | 8 ml |

**Table 2.7b Constituents of resolving gel for native gel**

### 2.6.3 Protein stability test, Protein Sequencing and Identification

The stability of recombinant IL-37 proteins were measured by Circular dichroism (CD) in the University of Glasgow by my colleague Najwa Hammed. CD machines obtained from Aviv Biomedical Ltd. (USA) for collecting data from 700 to 175nm wavelength. To check the protein composition, recombinant protein is unfolded in 6M Guanidine-HCl or 3% NaOH. The Number of amino acids. Theoretical pI and molecular weight can be automatically measured by the machine. Mass Spectrometry was also used to identify the recombinant protein and the protein sequencing analysis. The measurement and instrument management were assisted by the University of Glasgow.

### 2.6.4 Protein bioactivity test

To determine the bioactivity of the recombinant IL-37 a and b proteins the following procedure was followed: Murine macrophage (RAW 264.7) was seeded in 24-well cell culture plates at 1x $10^6$ per ml in DMEM media supplemented with 10% heat-inactivated fetal bovine serum, 100U/ml penicillin, and 100µg/ml streptomycin. Culture temperature is 37°C under 5% $CO_2$ culture condition in the cell culture incubator overnight. The cells were pre-incubated with different doses of IL-37a and IL-37b (50ng/ml and 100 ng/ml) and then added into plates and cultured at 37°C under 5% $CO_2$ condition for 2 hours. Furthermore, 200ng lipopolysaccharides (LPS, TLR4, Invivogen) was added to each well then incubated for 15 hours. The concentration of IL-6 in the cell culture supernatant was measured by an enzyme-linked immunosorbent assay (ELISA) kit (R&D system), The concentration of IL-6, the major cytokine induced by LPS the expression of which has been reported to be suppressed by IL-37 (Li *et al.*, 2015) was detected by ELISA. The relative bioactivity of the recombinant IL-37 a and b were detected relative to the ELISA commercial standard.

After endotoxin detection, the cleanness of recombinant IL-37a and b proteins were further checked by heat-inactivating the proteins. The test sample was boiled by 100°C water bath for 30 mins. 1x10^6 murine macrophage RAW cells were seeded in 24 wells cell culture plate; culture temperature is 37°C under 5% $CO_2$ culture condition in the cell culture incubator overnight. Furthermore, 50ng

of pure/ boiled recombinant IL-37a and b proteins were given to appropriate group. After incubating for 2h, 200ng LPS were given to all the test groups, then incubated for 15 hours. The gene expression of IL-6 in mRNA was measured by qPCR (Figure 2.4). The heat inactivated protein shows similar IL-6 level as only LPS group, indicates no contaminating PAMPS from the bacterial prep.

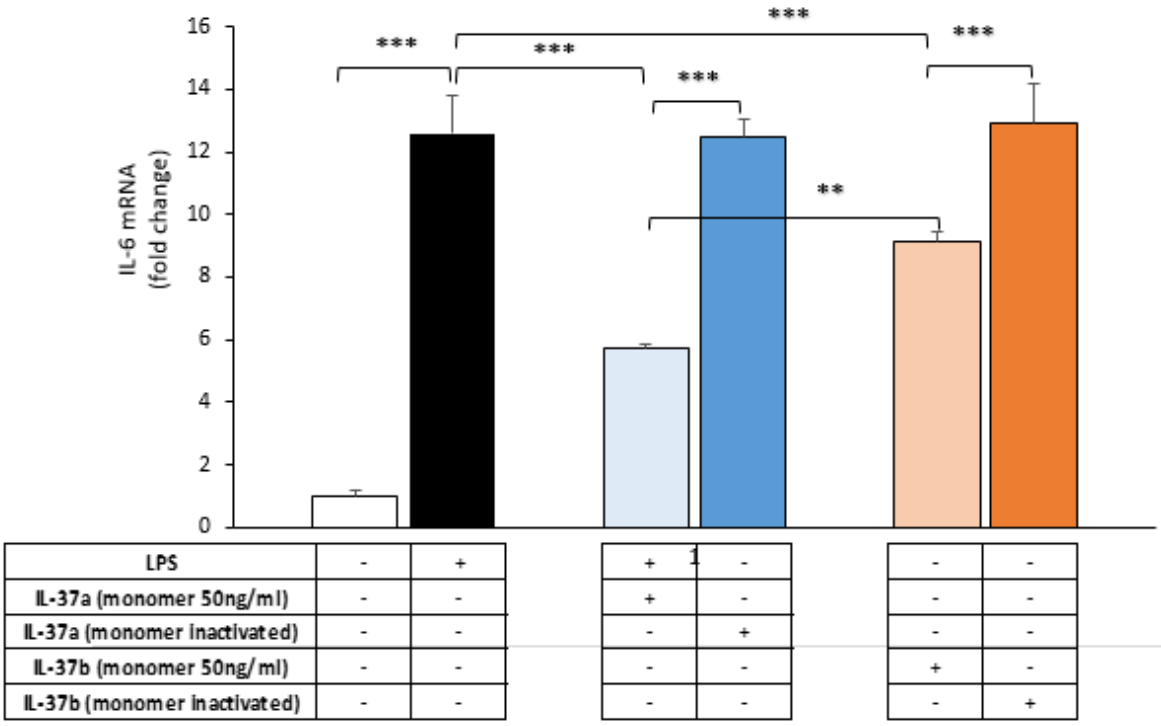



**Figure 2.4 Contaminating PAMPS checking by qPCR**

Murine macrophage RAW cells (1x10^6 cells/ml) were pre-treated with or without pure/ heat inactivated (100°C for 30 mins) recombinant monomer IL-37a and b stimulated with LPS, (200ng/ml) for 15 h. The gene expression levels of IL-6 in mRNA were measured by qPCR. Using student T-Test. Data are means ± SD, and are representative of three independent experiments. *P<0.05 and ***P<0.001 compared to controls.

## 2.7 Gene expression assay

### 2.7.1 Quantitative-PCR analysis

#### 2.7.1.1 RNA extraction

##### 2.7.1.1.1 Isolation of total RNA from cells

RNA of infected THP-1 and RAW cells was isolated by the Purelink RNA mini kit (Life technologies). After the cell lysis, test samples were vortexed in small Eppendorf tubes and homogenized by 21-gauge syringe for 10 times. After that, the purification process was performed following manufacturer's instructions. RNA concentration was measured in a Nanodrop 2000 (Thermo scientific). RNA samples were stored in -80°C for downstream process.

##### 2.7.1.1.2 Isolation of total RNA from tissues

To isolate tissue RNA, TRIzol Reagent (Invitrogen, UK) was used for extract total RNA. Murine tissues were preserved in RNAlater reagent for longer storage. Cut tissues into small fragments (10-100mg) then wash with 1ml cold PBS. After washing, dry tissue pieces, add 1ml TRIzol reagent per sample. Leave sample on ice for around 5 minutes then homogenize tissue sample at 19,000rpm by electric homogenizer (Thermo Fisher Scientific Inc., US). Incubate the sample at room temperature after homogenization then take the liquid face out, add same amount of ethanol to homogenized mixture contained RNA and mix well. Then centrifuge the mixture at 12,000rpm by mini centrifuge for 2 minutes. After that, the purification process was proceeding which follow the protocol from the kit. The impurities were extracted in Wash buffer (Life technologies) under 12,000rpm centrifuge condition, 4°C, then the sample RNA dissolved in DNase free water and collected in Eppendorf tubes. The concentrations of these RNA were measured by Nanodrop 2000 (Thermo scientific) within 260/ 280 ratio absorbance (1.8~2.1 was considered optimum). RNA extractions were kept on ice then put in -80°C for other in-depth process.

#### 2.7.1.1.3 RNA reverse-transcription to cDNA (RT-PCR)

After purification, the RNA concentration (unit: ng/μl) and the purity of RNA were confirmed by Nanodrop 2000 (Thermo Fisher Scientific). Additionally, the purified RNA was normalized into 10ng/μl, 100ng of total normalized RNA were used for the reverse reaction. The RNA was reversed transcribed into cDNA by the High-Capacity cDNA Reverse Transcription Kit (Life technologies). Prepare 10μl of reverse transcription master mix, each 10μl contains 10x RT PCR Buffer, 10x RT Random primers, 25x dNTP and 1μl of Reverse Transcription enzyme. Filling the rest with distilled water; mix the sample with master mix well by vortex. Reaction tubes were loaded into the thermocycler (96 wells thermocycler, Applied Biosystems) and run following the instructions from manufacturer. Keep the cDNA production in -20°C for further storage.

#### 2.7.1.1.4 Quantitative Real-Time PCR (qPCR)

The cDNA was measured and quantified by Quantitative Real-Time PCR (qPCR). Transcripts of cytokines-of-interest which expression was measured and calculated included IL-37 isoforms (a-e), iNOs and other related cytokines. Human/murine Gapdh was utilized as the housekeeping gene. The sequence of Forward and reverse primers (Integrated DNA Technologies, USA) are shown in the Table 2.8 and 2.9.

1 μl of cDNA which was obtained from the obtained from reverse transcription product was used as the template for Quantitative Real-Time PCR. 9μl of Fast SYBR green master mix reaction mixture (Thermo Fisher Scientific) was added into the reaction wells to make the total reaction volume 20μl. The reaction mixture contained 5μl Fast SYBR green master mix, 0.5μl Forward primer sequence (10 μM), 0.5μl Reverse primer sequence (10μM), 3μl Nuclease-free H2O and 1μl of cDNA template. The Quantitative Real-Time PCR was run in a 96 well (0.1ml) reaction plate for 40 cycles (Applied Biosystems Step One Plus Real-Time PCR system). Fold changes were calculated using the $2^{-\Delta\Delta}$Ct method. The samples were run in triplicates. Pro-incubation was performed at 95°C for 20s, amplification at 95°C for 15s and 60°C for 20s (40 cycles); and the annealing for 60s at 60°C.

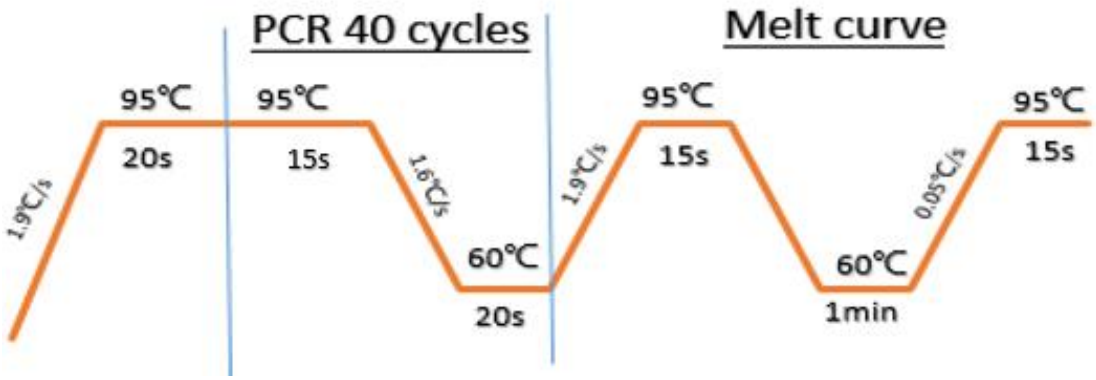



**Figure 2.5 qPCR process in this experiment**

| Primer name | Forward (5`-3`) | Reverse (5`-3`) |
|---|---|---|
| human GAP dh | GAG CCA CAT CGC TCA GAC AC | CAT GTA GTT GAG GTC AAT GAA GG |
| human IL-37a | GGG AAA CAG AAA CCA AAG GA | CCC AGA GTC CAG GAC CAG TA |
| human IL-37b | ATG TCC TTT GTG GGG GAG AAC TCA G | TGC TAT GAG ATT CCC AGA GTC CAG GAC C |
| human IL-37c | CCC CAC CAT GAA TTT TGT TC | CCT TTA GAG ACC CCC AGG AG |
| human IL-37d | AAC CCC AGT GCT GCT TAG AA | CCC AGA GTC CAG GAC CAG TA |
| human IL-37e | AAC CCC AGT GCT GCT TAG AA | CCT TTA GAG ACC CCC AGG AG |
| human INOs | ATG CCC GAT GGC ACC ATC AGA | TCT CCA GGC CCA TCC TCC TGC |
| human SIGIRR(IL-1R8) | TCA GTG GCT CTG AAC TGC AC | GTA CCA GAG CAG CAC GTT GA |
| human IL-18Rα | ACG CCG AGT TTG AAG ATC AGG GGT | CCC TGG GCA AAA TCT CCA CAG CA |
| human IL-1α | TGT ATG TGA CTG CCC AAG ATG AA | CTA CCT GTG ATG GTT TTG GGT ATC |
| human IL-1β | CAC GAT GCA CCT GTA CGA TCA | GTG GCT CCA TAT CCT GTC CCT |
| human IL-6 | AGC CAC TCA CCT CTT CAG AAC GAA | AGT GCC TCT TTG CTG CTT TCA CAC |
| human IL-10 | TCC CAG CCC AAG GGT AG | TCT GTC CCA TGA GCG TCT |
| human IL-12 | TCA AAC CAG AAC CAC CGA A | GCT GAC CTC CAC CTG CTG A |
| human IL-18 | ATC GCT TCC TCT CGC AAC AA | CTT CTA CTG GTT CAG CAG CCA TCT |
| human IL-33 | GGA AGA ACA CAG CAA GCA AAG CCT | TAA GGC CAG AGC GGA GCT TCA TAA |
| human IFN-γ | TCA GCT CTG CAT CGT TTT GG | GTT CCA TTA TCC GCT ACA TCT GAA |
| human TNF-α | CAG GGC AAT GAT CCC AAA GT | CAA TCG GCC CGA CTA TCT C |

**Table 2.8. Sequences of primers for the amplification of human genes by Quantitative Real-Time PCR** (qPCR).

| Primer name | Forward (5`-3`) | Reverse (5`-3`) |
|---|---|---|
| mouse GAP dh | GGC ATG GAC TGT GGT CAT GAG | TGC ACC ACC AAC TGC TTA GC |
| mouse iNOS | CCT GGT ACG GGC ATT GCT CC | GCT CAT GCG GCC TCC TTT GA |
| mouse SIGIRR | AGT CTC AGG TGG GTG GCA GT | GTC TCG GAG TTC TGG GTG AG |
| mouse IL-18Rα | CTT CGT CTT GGT GAG AAA AGA AAT A | TGT CAC AGC AGC ATA TTA CCT G |
| mouse IL-1α | GAC TTG CAC AGA TCA GTT TGT ATC | AGA GAC CAT CCA ACC CAG |
| mouse IL-1β | GGC AGG CAG TAT CAC TCA TTG | GAA GAG TGT GGA TCC CAA G |
| mouse IL-6 | GTT CTC TGG GAA ATC GTG GA | TTC TGC AAG TGC ATC ATC GT |
| mouse IL-10 | AAC AAA GGA CCA GCT GGA CAA C | CTT GAT TTC TGG GCC ATG CTT |
| mouse IL-12 | CAA CAT CAA GAG CAG TAG CAG | GTA CTC CCA GCT GAC CTC CAC |
| mouse IL-18 | GCC TCA AAC CTT CCA AAT CA | TGG ATC CAT TTC CTC AAA GG |
| mouse IL-33 | ACT ATG AGT CTC CCT GTC CTG | ACG TCA CCC CTT TGA AGC |
| mouse IFN-γ | CTG CGG CCT AGC TCT GAG A | ATG ACT GTG CCG TGG CAG TAA |
| mouse TNF-α | CCT GTA GCC CAC GTC GTA G | TTG ACC TCA GCG CTG AGT TG |

**Table 2.9 Sequences of primers for the amplification of murine genes by Quantitative Real-Time PCR** (qPCR)

### 2.7.2 Genotyping

#### 2.7.2.1 DNA extraction

Genotyping of UGLA0004 IL37a transgenic mice (strain C57BL/6) was performed by PCR. Mouse tail-tip biopsies (2mm) or ear pierced samples (2mm) were taken from the wild type mouse and transgenic mice then kept in -20°C for extraction. DNA was extracted from murine tail sample using 75µl Alkaline Lysis Buffer (containing 25mM NaOH, 40mM Tris-HCl and 0.2mM EDTA, PH=12). Cut sample into small pieces then mix the sample with Lysis Buffer in PCR Eppendorf tubes, heat the mixture on a heating block (Thermo Fisher Scientific, USA) at 95°C for 1 hour. During the lysis process, the Neutralizing Buffer (40mM Tris-HCl, PH=5) was prepared. Adding same volume of Neutralizing Buffer to each tube then measure the DNA concentration by Nanodrop 2000 (Thermo Fisher Scientific). Neutralizing Buffer was used as the blank for DNA measurement.

#### 2.7.2.2 PCR for genotyping

PCR was used for genotyping to distinguish the wild-type (WT) from IL-37a transgenic mouse by observing the specific bind of PCR products. Two different primer pairs were used (Taconic, Germany) (Table 2.10). Normalize the DNA level after measuring the DNA concentration. Add 5µl of normalized DNA template (80ng/µl) into PCR Eppendorf tubes. GoTaq Master Mix (Promega, UK) was used for the PCR process; Centrifuge the sample and master mix with MiniStar Micro centrifuge (VWR) till no liquid attach to the surface; perform the PCR reaction in the thermocycler (96 wells thermocycler, Applied Biosystems) as the progress below: heat the sample mixture at 94°C for 5 minutes, then 94°C 15 seconds, 55°C 30 seconds, 72°C 40 seconds, repeat 35 cycles; 72°C 10 minutes for final elongation. After the PCR process, put samples in -20°C for storage.

Mix the PCR products with 10x Bromophenol Blue Loading Buffer (Invitrogen, UK). Add 20µl of mixture into separate wells of 1.5% Agrose in 1x TAE gels with additional ethidium bromide 0.03 µl/ml. DNA ladder (Invitrogen, UK) was used for checking the molecular weight expected fragments. Both WT and Tg samples, negative control (without DNA template) and ladder were loaded in 1x TAE buffer in an electrophoresis (Sigma) tank using Basic Power Supply Instruction (Bio Rad,

UK) at 100mV for 40-50 minutes. Image was determined by Gel Logic 200 Imaging System (Kodak, USA) under UV light, explore time is 2 seconds. The typical binds for WT and IL-37aTg mouse present in Figure 3.4.

| Test gene | primer name | primer sequence (5`-3`) | Expected Fragments |
|---|---|---|---|
| **SOP 13021** | 2970_5 caggs3: | ATC CAC TTT GCC TTT CTC TCC | 379dp |
| | 13021_1: | ATG GAT GAC TTT GTC CTT TAT CC | |
| **SOP 13022** | 13022_3 | GCA ATT GTA ATG AGC CTG TTG G | 401dp |
| | 6166_41 hGH pA 3´4: | CCT TAA TTA AGT CGA CGG ATC G | |
| **control** | 1260_1 | GAG ACT CTG GCT ACT CAT CC | 585dp |
| | 1260_2 | CCT TCA GCA AGA GCT GGG GAC | |

**Table 2.10 IL-37aTg mouse PCR primer pair sequences**

## 2.8 Bacteria culture and infection

### 2.8.1 SL1344 culture

Salmonella strain SL1344 was utilized as the infection microorganism in this project. The SL1344 colonies were cultured in a LB broth (Luria Bertani broth medium: 0.5% yeast extract, 1% tryptone, 1% NaCl) agar (with 1% agarose) plate before experiments. One single colony was collected and cultured in a 5ml LB broth culture universal in an incubate shaker under 180rpm, 37°C. Then the bacteria mixture was diluted 1:10 in LB broth and incubate in a stationary 37oC incubator over night before infection. OD level of SL1344 dilution was measured by photometer, appropriate absorbance level should be between 0.5-0.6. After OD measurement, the bacteria culture was diluted again in 1:6 in culture media RPMI1640 with 3% FBS for cell infection.

### 2.8.2 BRD509 culture

An attenuated strain of *Salmonella* (BRD509) was used in the *in vivo* test of toxicity during optimization of the experimental condition. Similar as SL1344 strain culture, the signal colony of SL BRD509 was transferred into 5ml of the LB broth with a sterile stick and incubated in a shaker under 180rpm, 37°C for 4 hours. Then 50ul of bacteria culture were added into 500ml of pre-auto-cleaved LB broth and stable incubate in 37°C incubator for at least 6 hours. After 6 hours` incubation, the OD level of BRD509 dilution was measured by a photometer (absorbance level should be between 0.5-0.6). With the standard curve of BRD 509 CFU versus OD reading, appropriate volume of bacteria culture was collected for different purpose of experiment. Bacteria were centrifuged under 5000xg for 5 minutes, and after the supernatant was discarded, bacteria pellets were resuspended in PBS for *in vivo* oral infection.

### 2.8.3 Human and murine macrophage *S. Typhimurium* infection

At the beginning of Salmonella infection, 200µl of SL1344 dilution (1:6) was pipetted into a 24 wells tissue culture plate. In comparison, 200µl of RPMI 1640 media with 3% heat inactivated FBS and 2mM L-glutamine also added into the culture plate as a negative control. Then the plate was placed put in 37°C, 5% $CO_2$

incubator to wait for the infection to be established. After 1h, all the supernatant was removed and replaced with 400µl fresh RPMI 1640 media with 3% heat inactivated FBS and 2mM L-glutamine as well as additional 50µg/ml antibiotic (Gentamycin). The purpose of additional Gentamycin was to eliminate extracellular SL1344 which have not invaded the macrophages. The plate was keeping in the bacterial incubator during the infection progress for several time points at 37°C, 5% $CO_2$ condition.

Once the time-points had elapsed, the supernatant was collected then stored in -20°C freezer immediately in order to kill any residual salmonella which may not have been killed by antibiotic. Supernatant was used to test the production of Nitric Oxide (NO) by the Griess diazotization reaction, and to detect and quantify the concentration of pro-inflammatory protein level (human/mouse IL-6) by Enzyme-Linked Immunosorbent Assay (ELISA). The adherent cells which contain live bacteria were harvested using 2% Trition-X100 then prepared for bacteria CFU counting. Furthermore, the 1:6 SL13344 dilution was also used for other in-depth experiments and RNA extraction under same culture condition. At the end point of infection, the macrophages which attach to the bottom of each well were harvest by cell lysis buffer then put on ice to direct purify RNA or further processing.

### 2.8.4 Dilution plate for bacteria counting

Infected cells were harvested with 2% Triton-X100 in PBS and lysed using a sonic water bath (DAWE instrument, UK) to release the intracellular salmonella which contained in the macrophage for spot plate analysis. Cell lysate of SL1344 was diluted 1:10, 8 times in a 96 wells cell culture plate. 10µl of each dilution was dropped in triplicates on LB agar plate and incubated at 37°C for 24 hours. Colony forming units (CFU) were counted and calculated with dilute equations, in order to find out the relative number of SL1344 which infected THP-1 and RAW macrophages.

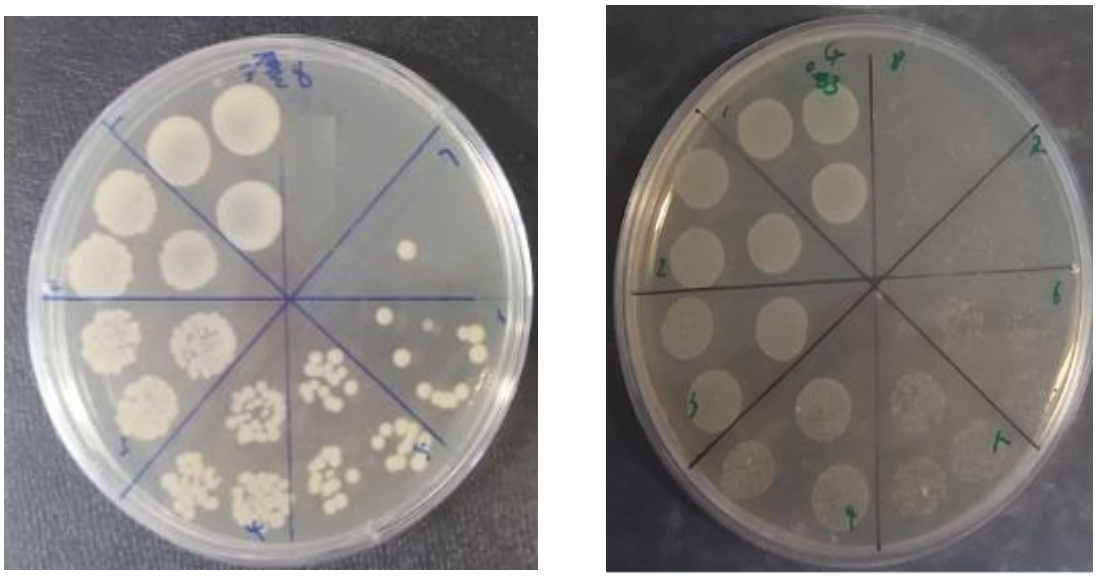


**Figure 2.6 Bacteria spot plates of salmonella strain 1344 and BRD509**

### 2.8.5 Nitric oxide (NO) production test

Griess Reagents kit (Progema) was used for the detection of Nitric oxide (NO) expression. Reagent I and II were put in room temperature for 30 minutes before detection. $NaNO_2$ (1M) was utilised as the test standard and dilute with cell culture media. Test samples come from the supernatants of cultured cells and put in 96-well ELISA plate 50µl per well in Triplicate. In addition, 50µl of Reagent I and II were added into each well, separately. Optical densities (OD) of standards and experimental samples were read at 550nm wavelength using a 96 wells plate reader (Dynex technologies). NO concentrations were plotted against a standard curve set up which compare with the standard curve of the Griess kit in order to account for calculation errors when reading. Then the concentration of each experimental sample can be calculated from the standard curve.

### 2.8.6 Tool-like receptor (TLR) stimulations

RAW 264.7 macrophages were cultured at $1.2x10^5$ cells in 24 wells cell culture plate, the cells were seeded overnight period of the stimulation. THP-1 cell differentiates into macrophage-like cells. They were seeded at $1.2x10^5$ cells in 24 wells in 4 days before stimulation with PMA then the culture media was replaced by the fresh culture media 48 hours before stimulation in order to remove the effect of PMA. Different doses of TLRs: lipopolysaccharide (LPS, TLR4, Invivogen), synthetic triacylated lipopeptide (PAM3cSK2, TLR1/2, Invivogen) and Flagellin from *S. Typhimurium* (FLA-ST, TLR5, Invivogen) were added in both cell lines and incubated with different time points. Cell lysate and supernatant were harvested then collected at appropriate time according to experiment requirement. The supernatant was used for NO detection and ELISA test, and RNA was purified form the cell lysate as a template for qPCR and further analysis.

## 2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatants of cultured cells were analysed for the levels of IL-6 using the Human IL-6 Quantikine ELISA Kit (R&D systems) and Mouse IL-6 Quantikine ELISA Kit (R&D systems) following the manufacturers. The wells were coated with a polyclonal antibody specific for human IL-6 with an ELISA cover sealer and incubated in room temperature overnight. After overnight incubation, the coating buffer was discarded and washed with washing buffer 3 times, 100µl of assay diluent (4 times) and standard were added in appropriate wells for 2 hours of incubation in room temperature. Moreover, after another 4 times washing steps to remove unbound composites, 100µl of detection antibody solution was added into both IL-6 standard and test samples then incubate for another 2 hours. After this incubation, washing the plate again to remove excess polyclonal antibody that has not bound before additional Conjugate (HRP conjugated) and cover with a new plate sealer for 2 hours` incubation. After the final washing steps, 100µl Substrate Solution (TMB, 3, 3', 5, 5'-tetramethylbenzidine) was added to test peroxidase activity. ELISA plate was cultured in dark protected from light for 20 minutes in this step to avoid the photolysis of the reagents. Then adding 50 µL of Stop Solution (0.2M sulfuric acid) to each well to stop the reaction. The ELISA plate was reading by a 96 wells plate reader (Dynex technologies) at 450nm wavelength. Optical densities (OD) of experimental samples were then plotted against human/ mouse IL-6 standard values for creating the IL-6 standard curve. IL-6 concentration (pg/ml) was calculated by the standard curve in order to know the extracellular protein (human/mouse IL-6) level in each sample.

## 2.10 siRNA

Small interfering RNA (siRNA) molecules against human IL-37 and its isoform IL-37a were designed and used to knockdown the expression of the proteins-of-interest proteins (IL-37a mRNA GeneBank accession mumber: NM_173205.1). Custom siRNA oligos were synthesized and obtained from Invitrogen Life Technologies (UK). siRNA sequences of IL-37 and IL-37a are shown in Table 2.11 The RNA interference (RNAi) negative control is Silencer® select negative control#1 siRNA obtained from Thermo Fisher Scientific as a nontargeting siRNA control, 5nM with Nuclease-free water. For the siRNA transfection experiment, human THP-1 cells were seeded in 24-well cell culture plate at $1.2x10^5$ cells/ ml per well then stimulated with additional 50ng of TPA/PMA (Sigma-Aldrich) and incubated for 48 hours before transfection. After incubation, suspension cells were removed by washing with complete RPMI twice. SiRNA sequence for IL-37 IL-37a and negative control was mixed by Lipofectamine® RNAiMAX Reagent (Cat: 13778-100, Thermo Fisher Scientific). RPMI medium was used with additional 10% FCS (fetal calf serum) and 2mM L-glutamine to avoid the infection of antibiotics. After incubation for 48 hours, cells were stimulated by extra 100ng LPS after washing and changing into new complete culture medium. Cells were collected at 6, 12, 18 hours later by lysis buffer and the efficiency of IL-37 and IL-37a SiRNA was confirmed by qPCR analysis.

| Target gene | primer position | primer sequence (5`-3`) |
|---|---|---|
| **IL-37** | Target all isoforms | UCA AGG AUG AGG CUA AUG CUU |
| | | CAA UGU GUU UCC UGU UCU CUU |
| | | UUA CAA UUG CAG GAG GUG CUU |
| | | UUA UCC UUG UCA CAG UAG AUU |
| **IL-37a** | 5#（target 16-38）: | GAG GGA AAC AGA AAC CAA AUU |
| | | UUU GGU UUC UGU UUC CCU CUU |
| | 21#（target 35-57）: | GGA AAG AAC AGC UUU AAG AUU |
| | | UCU UAA AGC UGU UCU UUC CUU |

**Table 2.11 siRNA primer sequence**

## 2.11 Transcriptome analysis

Transcriptome analysis was performed on transgenic IL-37a and b mice (transgenic mice with CMV promoter) splenocytes. Splenocytes of WT C57BL6/J and IL-37a/bTg mice were seeded in 24 wells cell culture plate in the density of $1x10^6$/ml. These cells were stimulated with 500ng/ml LPS for 4 hours. Total RNA from each sample was isolated and purified. Construction of cDNA libraries and next-generation sequencing was performed.

Raw data from RNA-seq were analysed for each group (WT, IL-37aTg mice and IL-37bTg mice, n=3). The average RPKM (Reads Per Kilobase Million) per group was estimated. Moreover, the p-value and fold-changes were analysed for comparisons between: IL-37a vs. WT and IL-37b vs. WT. Using a cut off values (p value< 0.05, fold-change $\geqslant$1.5), we detected the differential expressed genes compared IL-37a and bTg vs. WT.

Bioinformatics analysis: Area-proportional Euler diagrams were generated using the BioVenn© tool (http://www.biovenn.nl/). The genes in IL-37a/bTg mice vs. WT specific and common area of the Venn diagram were analysed for the detection of TLR-related signalling pathways which affect by IL-37a or b specifically or commonly by String DB software, Gene ontology (GO) and KEGG pathways database.

## 2.12 Animal work

The experimental group size was determined by disease incidence and from the expected difference among experimental groups from preliminary experiments or from literature search wherever possible, also taking into account resources available and ethical animal use. A power of 0.8 was used to calculate the experiment group sizes (Festing and Altman, 2002, Charan and Kantharia, 2013).

### 2.12.1 Lipopolysaccharide (LPS) challenge

Mice are grouped and injected intraperitoneally with a sub-lethal dose of LPS (Sigma-Aldrich, UK) in 200 µL PBS by a 26-gauge needle. Final LPS dose is based on the weight of each mice in LPS treat group at 1µg/g body weight (around 150ug/mice) and the control group were given same volume of PBS. Mice are kept in appropriate condition for 18 hours after injection and was killed by $CO_2$ under ethical protocol. A 500µl blood sample is taken from heart were collected for serum isolation and spleens were harvest and kept in complete RPMI media on ice. Blood samples are analysed for the expression level of IL-37 by ELISA, RNAs were isolate from spleen for qPCR measurement.

### 2.12.2 S. *Typhimurium* (BRD509) infection model

S. *Typhimurium* (BRD509) were cultured overnight with shaking at 200 RPM before the infection model set up. These bacteria were further diluted in PBS in 1x10^8 CFU/ml. Mice are grouped and given 5% Sodium carbonate (Sigma-Aldrich, UK) in 100ul/mice by a bulb-tipped 22G gavage needle and a 1 mL syringe to neutralize stomach acid. Then mice were challenged by oral infection with 200 ul of $1x10^8$ CFU/ml BRD509, same amount of Sodium carbonate and PBS were given to the control mice orally.

we use a 0-4 scoring system to rate the severity of diarrhea based on the gross appearance of the colon at necropsy. A normal feces was given a score as 0. If the cecum was shrunken and white it was scored as 1. We gave 4 score to a mouse that had a shrunken cecum and a distal colon that was fluid-filled with little or no formed stool. The whole process was checked by the stuff of CRF (Table 2.12).

The mice were kept for 6 and 8 days and killed by $CO_2$ under ethical protocol. A 500µl blood sample is taken from heart were collected for serum isolation, liver and drain lymph nodes were collect in PBS for bacteria counting, and spleens were harvest and kept in complete RPMI media on ice. Serum samples are analysed for the NO level, bacteria from liver and LN were counted by spot plate, and RNAs were isolate from spleen for qPCR measurement.

| Grade | Criteria |
|---|---|
| 0 | Normal colon with normal formed feces in the distal colon or rectum. |
| 1 | Cecum that is smaller than normal with areas of white and/or gas bubbles in the lumen; the rest of the colon is normal. |
| 2 | Severe typhlitis (white, shrunken cecum) with involvement of the proximal colon so that it is fluid filled; formed stools in distal colon. |
| 3 | Grade 2 plus fluid throughout most of the colon with soft feces in the distal colon. |
| 4 | No formed feces in the entire colon and the cecum white and shrunken; colon filled with clear or bloody mucus. |

**Table 2.12 Scoring system for grading diarrhea**

## 2.13 Statistical analysis

The statistical evaluation of the experimental data was performed using GraphPad Prism v5.0a (GraphPad Software Inc.). Depending on the normal or skewed distribution of the data, either a T-test or a Mann-Whitney test was used respectively when comparing two sets of data. For comparison of more than two groups, a one-way ANOVA (or non-parametric equivalent) method was utilized. Any p-value of <0.05 was considered statistically significant. The two-tailed value of significance was set at $p \leq 0.05$ (*), 0.01 (**) or 0.001 (***) for describing group differences. Data were expressed as means with ± standard error of mean (SEM), unless otherwise indicated.

# Chapter 3

# Generation of recombinant IL-37 proteins and IL-37 transgenic mice

# 3. Generation of recombinant IL-37 proteins and IL-37 transgenic mice

## 3.1 Introduction

In order to understand the biological function of IL-37a and the functional difference between IL-37a and b, we sought firstly to produce recombinant IL-37a and b proteins. Furthermore, to study the function of human IL-37a *in vivo* in diseases, we generated human IL-37a transgenic mice in our lab. Moreover, to reveal if IL-37a and b signal via the same or different receptor, we also obtained and breed the IL-37 receptor IL-1R8 deficient mice (Garlanda *et al.*, 2004).

Bacterial protein expression system is widely employed to produce recombinant protein for the purpose of research and drug development (Joseph *et al.*, 2015). The His-tagged recombinant human IL-37b proteins have been sucessfully produced in *E. coli* (Gu *et al.*, 2015). IL-37b proteins expressed in *E. coli* can be soluble located in the cytoplasm or insoluble in inclusion bodies (Yang *et al.*, 2011; Singh *et al.*, 2015). The soluble proteins can be directly purified to produce the natural bioactive IL-37 (Gu *et al.*, 2015). The insoluble proteins in the inclusion bodies have to be made soluble first by denaturing the proteins with detergent and then purifing them. The purified denatured proteins then need to be renatured by the removeal of the detergent from the protein in order to regain their bioactivity (Yang *et al.*, 2015). After expressed in *E. coli*, the His-tagged IL-37b proteins can be initially purified by metal-affinity chromatography, for instance, Nickel affinity chromatography (Gu *et al.*, 2015). The His-tag of proteins can bind to the Ni-NTA agarose resin in the low dose of imidazole and can be eluted by increasing the imidazole concentration. Resins, such as Ni-NTA Superflow Agarose provide greater capacity for His-tagged protein purification, can bind up to 80mg of His-tagged protein per ml of agarose beads (Stuehr *et al.*, 1991; Kaur and Reinhardt, 2012). However, the Nickel purified proteins from *E. coli* can only be 80-85% pure and will be still contaminated with bacterial proteins and lipopolysaccharide (LPS). The proteins need to be further purified by different approaches of chromatography, including gel filtration and ion-exchange. The LPS can be removed effectively from purified proteins from *E. coli*, including IL-37b by EndoTrap® affinity chromatography (Guillou *et al.*, 2015. Gu *et al.*, 2015).

While the recombinant IL-37b has been produced successfully, there is no report on IL-37a purificaiton, and no commercial IL-37a is avilable at the moment.

Recently, it has been reported that natural IL-37b proteins can form homodimer (Ellisdon *et al.,* 2017). The study of IL-37b crystal structure identified that the number 85 amino acid, tyrosine (Y85) in IL-37b protein sequence is responsible for the dimerisation of IL-37b (Ellisdon *et al.,* 2017). The replacement of the tyrosine (Y) with alanine (A) by point mutation in IL-37b cDNA has effctively prevented IL-37b dimerization (Ellisdon *et al.,* 2017, Eisenmesser *et al.,* 2019). As with the natural IL-37b, the mutant His tagged-IL-37b has been produced in *E. coli* and purified by the Ni-affinity chromatography and gel filtration. The mutant IL-37b proteins are existed in the monomer form. More importantly, the IL-37b monomer, rather than homodimer, has bioactivity (Eisenmesser *et al.,* 2019). However, it is unknown whether the natural IL-37a can also form homodimer and if this affects its function.

While IL-37 gene was lost in mice during evolution, the IL-37 receptors, both IL-1R8 and IL-18Ra chain, reamin intact and functional (Nold-Petry *et al.,* 2015). As with most cytokins, human IL-37b is also functional in mice (Nold-Petry *et al.,* 2015, Nold *et al.,* 2010). Thus, mouse has provided a unique model to study human IL-37 function *in vivo*. Human IL-37b transgenic mice have been generated that are critical in the understanding of the importance of human IL-37b in homostasis and disease (Nold *et al.,* 2010, McNamee *et al.,* 2011, Teng *et al.,* 2014, Lunding *et al.,* 2015).

The IL-1R8-deficient (IL-1R8KO) mice have been generated by several research laboratories (Wald *et al.,* 2003, Garlanda *et al.,* 2004). Using this strain of mice, IL-1R8 had been defined as a signaling receptor in IL-37b receptor complex (Nold-Petry *et al.,* 2015). It will be intriguing to know if IL-37a signal via the same receptor as IL-37b.

As the first step toward the understanding of the biological function of IL-37a and what is the difference between IL-37a and b in immunity and disease, in this chapter, I sought first to determine the dimerisation of IL-37a and then to produce both natural and mutant IL-37a and b proteins in *E. coli* system. Furthermore, the human IL-37a transgenic mice were also generated to study IL-37a *in vivo*. In order

to identify IL-37a receptor, we also obtained the IL-37 receptor (IL-1R8) knockout mice. The aims of the chapter were:

1. Determination the dimerisation of IL-37a.

2. Expression and purification of natural and mutant IL-37a and b proteins in *E.coli.*

3. Generation and breeding of IL-37a transgenic and IL-37a receptor (IL-1R8) knockout mice.

## 3.2 Results

### 3.2.1 Comparison of IL-37a and IL-37b in the amino acid level and biochemical parameters

In order to understand the biochemical variations between these two IL-37 isoforms, the differences between IL-37a and b in amino acid level were compared using analysis platform Protparam (Boraschi *et al.*, 2011). Analysis results were showed in table 3.1.

The IL-37a has 9 positively charged arginine and 22 lysine residues; the isoform b has 5 positively charged arginine and 20 lysine, which comprise 16.2% and 11.5% of total proteins in the two isoforms, respectively. The percentages of negative charged amino acids (aspartate and glutamate) in the two isoforms are similar, 11.5% in IL-37a and 12.9% in IL-37b. There was no dramatic difference in the percentage of aliphatic amino acids between these two isoforms. In general, the differences in amino acid have led to a clear difference in isoelectric point (pI) between IL-37a and b (9.23 vs 6.09, respectively). These variations may make them different in protein charge, solubility and stability which may influence protein purification and their bioactivity.

| | IL-37a | | IL-37b | |
|---|---|---|---|---|
| Amino acids | Number of amino acids | Percentage of amino acids % | Number of amino acids | Percentage of amino acids % |
| Ala (A) | 12 | 6.2 | 13 | 6 |
| Arg (R) | 9 | 4.7 | 5 | 2.3 |
| Asn (N) | 9 | 4.7 | 10 | 4.6 |
| Asp (D) | 9 | 4.7 | 11 | 5 |
| Cys (C) | 7 | 3.6 | 8 | 3.7 |
| Gln (Q) | 6 | 3.1 | 7 | 3.2 |
| Glu (E) | 13 | 6.8 | 17 | 7.8 |
| Gly (G) | 11 | 5.7 | 13 | 6 |
| His (H) | 5 | 2.6 | 6 | 2.8 |
| Ile (I) | 8 | 4.2 | 8 | 3.7 |
| Leu (L) | 15 | 7.8 | 17 | 7.8 |
| Lys (K) | 22 | 11.5 | 20 | 9.2 |
| Met (M) | 4 | 2.1 | 6 | 2.8 |
| Phe (F) | 11 | 5.7 | 12 | 5.5 |
| Pro (P) | 11 | 5.7 | 17 | 7.8 |
| Ser (S) | 21 | 10.9 | 25 | 11.5 |
| Thr (T) | 4 | 2.1 | 4 | 1.8 |
| Trp (W) | 2 | 1 | 3 | 1.4 |
| Tyr (Y) | 3 | 1.6 | 3 | 1.4 |
| Val (V) | 10 | 5.2 | 13 | 6 |

**Table 3.1 Differences between IL-37a and IL-37b in the amino acid level** (data from https://web.expasy.org/protparam).

| Parameter | IL-37a | IL-37b |
|---|---|---|
| Molecular weight (kDa) | 21.5 | 24.1 |
| Theoretical iso electric point (pI) | 9.2 | 6.1 |
| Number of amino Acids | 192 | 218 |
| negatively charged amino acids (%) | 11.5 | 12.9 |
| positively charged amino acids (%) | 16.2 | 11.5 |
| Nonpolar aliphatic amino acids (%) | 34.8 | 33.6 |
| Polar amino acids (%) | 27.6 | 28.9 |
| Aromatic amino acids (%) | 8.4 | 8.3 |

**Table 3.2 Comparison of biochemical parameters between IL-37a and IL37b proteins** (data from http://web.expasy.org/compute_pi).

### 3.2.2 Production of recombinant IL-37a and b protein

In order to investigate the biological function and difference between IL-37a and b *in vitro* and *in vivo*, large quantities of recombinant protein are needed. Therefore, the full-length IL-37a and IL-37b recombinant proteins were produced in *E. coli* system and purified with several purification methods. The working flow chart of recombinant human IL-37a and b protein production process is shown as Figure 3.2.

#### 3.2.2.1 Plasmid construction for the expression of IL-37a and b in *E. coli* system

The plasmid encoding His-tagged full-length recombinant human IL-37a and IL-37b were constructed by Dr Jiangning Liu in Dr Xu's lab (unpublished results). Briefly, human IL-37a and b cDNAs were cloned between Sal I and Xba I sites of pET- 21 (+) and pET- 28 (+) vector, respectively, and fused with hexahistidine cDNA. The His-tagged recombinant IL-37a and b were expressed in the plasmids with the T7 promoter (Figure 3.3). The recombinant plasmids were transformed into *E. coli* strain BL21(λDE3) and the successful transformed bacteria were selected by LB agarose plates containing appropriate antibiotic (Ampicillin and Kanamycin for IL-37a and b, respectively).

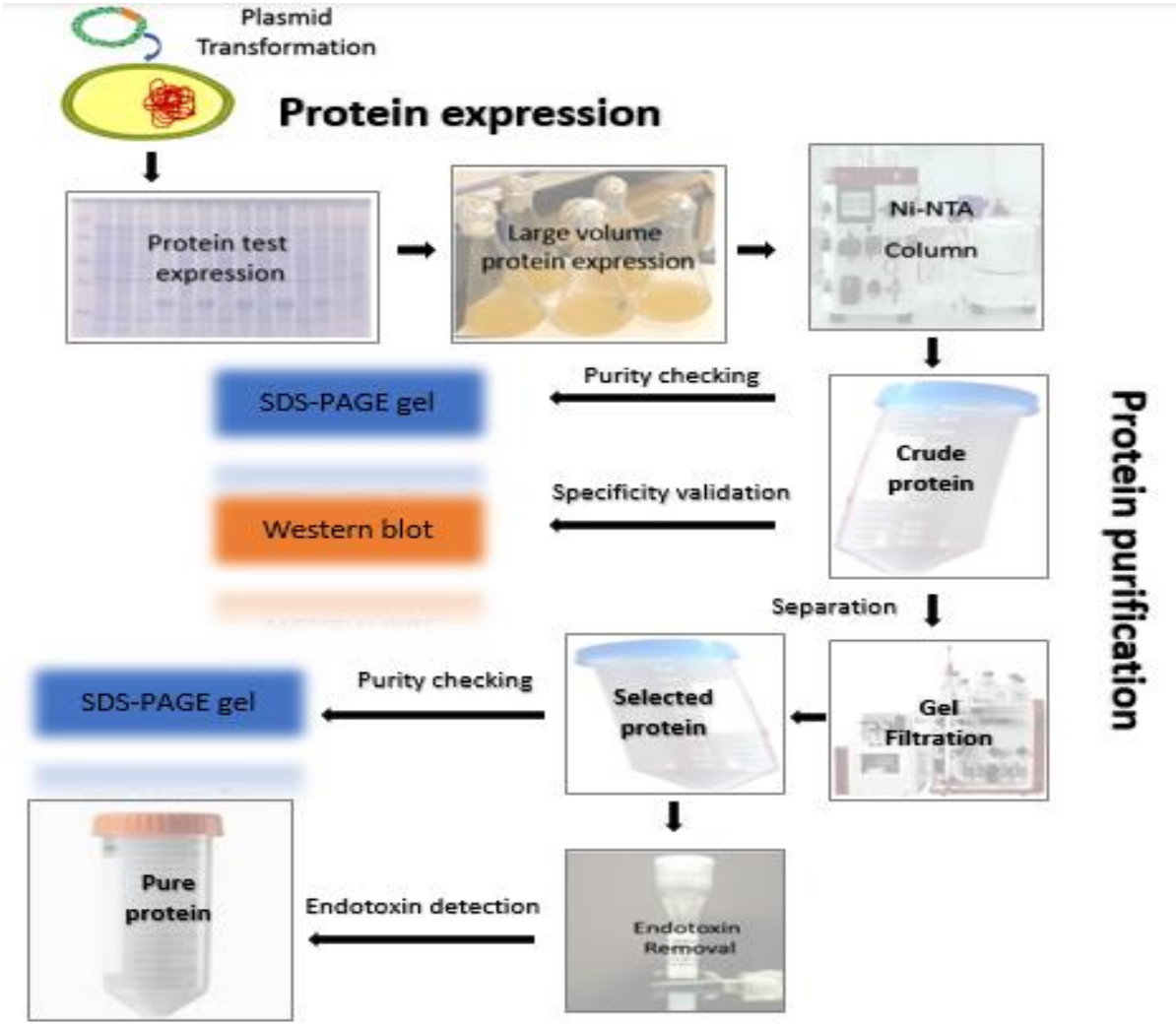



**Figure 3.1 Flow chart of IL-37a and b recombinant protein expression and purification**

The plasmid vectors for the expression of hexahistidine His-tagged recombinant IL-37a or b protein were transformed into host *E. coli* BL21 (DE3). The protein expression was inducible by adding Beta-galactosidase expression inducer — IPTG. The recombinant His-tag proteins are purified initially by Ni-NTA column followed by gel filtration system. The purity and specificity of the protein were subsequently confirmed by SDS-PAGE gel and western blotting after endotoxin removal by Endotrap® column.

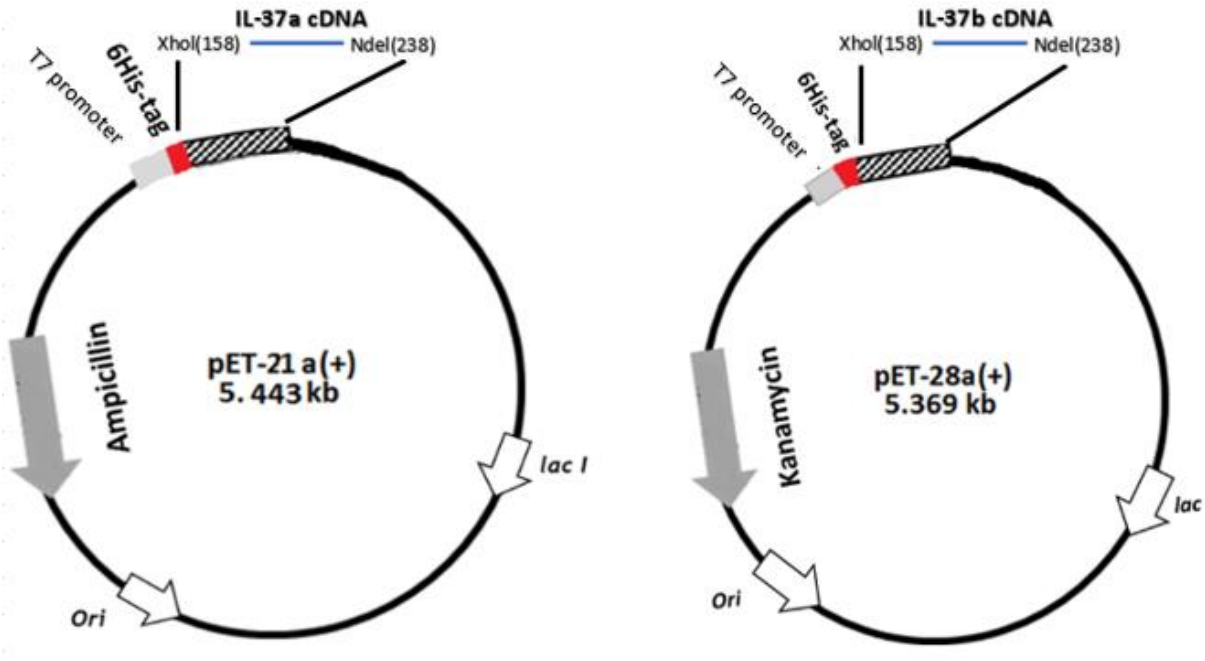



**Figure 3.2 Schematic representation of IL-37a/b construct**

The PET-21a (+) and PET-28a (+) vectors were used to express recombinant IL-37a and IL-37b, respectively. These two vectors can express His-tag fusion proteins with T7 promoter. In these two vectors, Ori means the origin of replication; dark grey areas are the antibiotic resistant gene coding sequence (Ampicillin resistant gene in PET-21a, Kanamycin resistant gene for PET-28a); light grey areas are T7 promoters; the red areas are the coding sequences of 6His-tag, IL-37a and b cDNAs were inserted into the two plasmids between the Nde I(158) and Xho I(238) sites.

### 3.2.2.2 Test expression of IL-37a and b

We firstly tested the expression of recombinant human IL-37a and b proteins in *E. coli* strain BL21 (λDE3). Briefly, the host *E. coli* strains carrying the plasmid vectors expressing IL-37a and b, respectively, were cultured in LB medium in the existence of ampicillin or kanamycin for recombinant IL-37a and b expressions, respectively. IPTG was added into the cultures as the protein expression inducer in the Log phase of bacterial growth. We firstly determined the kinetics of expression of recombinant IL-37a and b in *E. coli* with or without IPTG induction. The protein expression levels of IL-37a and b in the transformed bacteria were determined by SDS-PAGE. The SDS-PAGE result in Figure3.3 showed that the optimized induction time for both IL-37a and b recombinant protein expression was between 4-5 hours, with higher specific protein (around 25kD) expressions but relatively lower unspecific protein expression in *E. coli*. However, after 5 hours induction, the expression rate of our recombinant proteins was decreased.

Since recombinant protein produced in *E. coli* system can be soluble or insoluble (Sørensen and Mortensen, 2005). I firstly checked the solubility of the expressed IL-37a and b proteins by *E. coli*. The *E. coli* expressing recombinant IL-37 proteins were cultured in shaking incubator with IPTG induction for 5 hours. After induction, the bacterial pellets containing recombinant IL-37a and b were collected by centrifugation and then re-suspended in lysis buffers containing protease inhibitors and lysate by sonication. The soluble and insoluble parts of protein produced by the *E. coli* were separated simply by centrifugation. The IL-37 protein location in the supernatant (soluble) and cell pellet (insoluble) were checked by SDS-PAGE. Most of the recombinant IL-37 proteins were detected in the liquid phase of cell lysate (result not shown). Thus, the majority of the IL-37a and b recombinant proteins in our expression system were soluble.

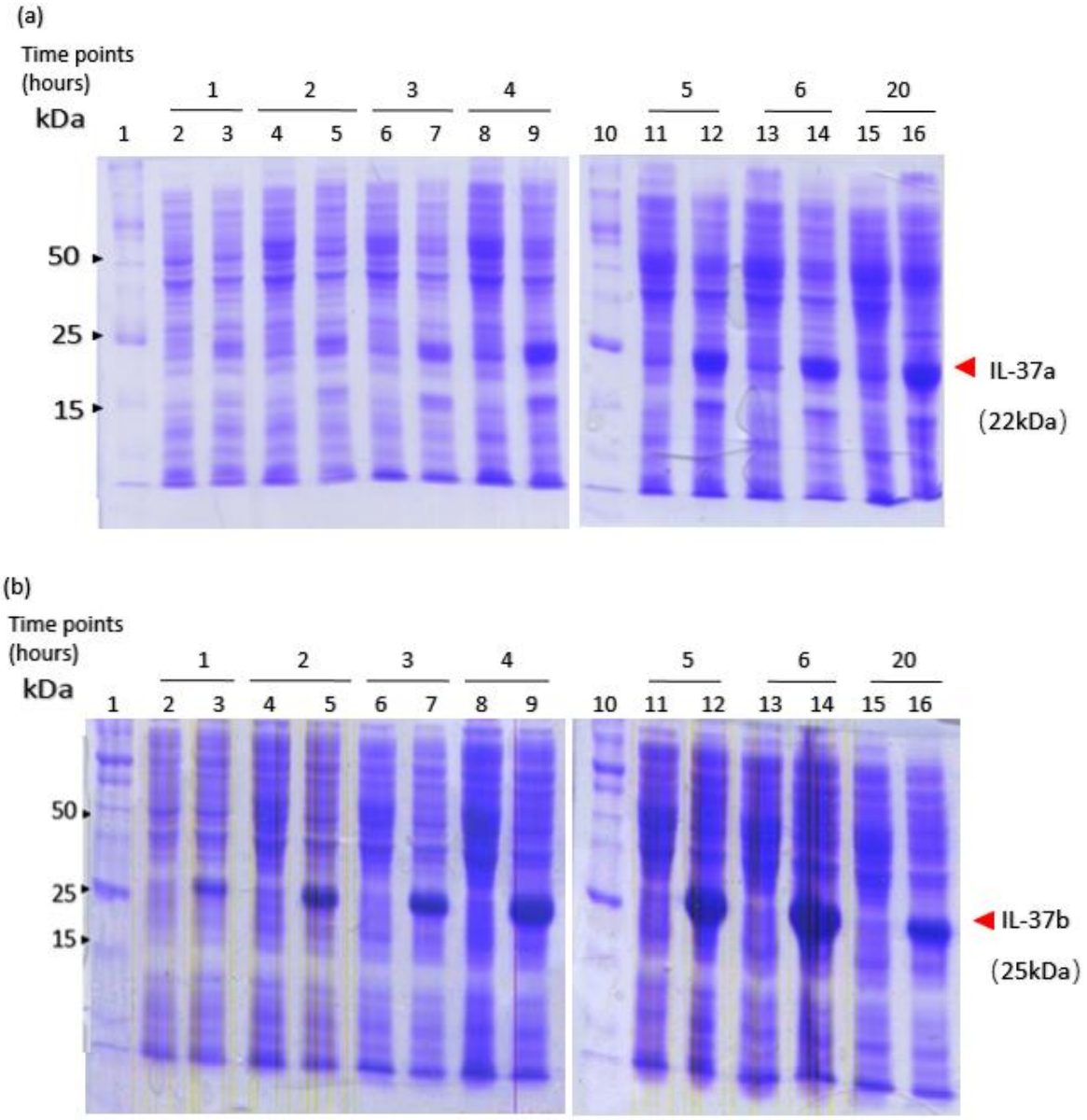



**Figure 3.3 Test expression of recombinant IL-37a and b in E. Coli**

The kinetic expressions of IL-37a and b proteins in *E. coli* (BL21) were induced by 1mM IPTG at 37°C up to 20 hours. After induction, total bacteria were lysed to get the soluble proteins. The proteins were separated by 15% SDS-PAGE and stained with Coomassie blue. a) The test expression of IL-37a recombinant protein. b) The test expression of IL-37b recombinant protein. The line 1 and 10 of these two gels are the protein kDa markers. Line 2,4,6,8,11,13,15 are the protein expressions of BL21 without IPTG induction in different time points (as control group). Line 3,5,7,9,12,14,16 are the protein expressions of test group with IPTG induction. The result is representative of at least three experiments.

### 3.2.2.3 Purification of recombinant IL-37a by Ni-NTA chromatography

The recombinant His-tagged IL-37a proteins expressed in the *E. coli* host were initially purified by Ni-NTA affinity purification. As the six-histidine tag of the recombinant IL-37a and IL-37b protein can bind to $Ni2^{+}$ in the Ni-NTA column (Stuehr *et al.,* 1991; Kaur and Reinhardt, 2012). The soluble proteins that contain the IL-37 proteins in the bacterial lysis were loaded onto the Ni-NTA column (Figure 3.4a). The unbound proteins were eluted from the column and the non-specifically bound proteins were washed away from the column using Elution buffer (containing imidazole, the flow rate was 2ml/min) until no protein could be detected in the flowed-out fraction (Figure 3.4a). The His-tagged IL-37a proteins were eluted from the column, shown as a sharp protein peak, at 74% of the elution buffer level within 120-190 min after loading the sample (Figure 3.4a).

The fractions from T7-T14 within the protein peak were collected as recombinant IL-37a protein fractions. To evaluate the success of the affinity purification of His-IL-37a protein by Ni-NTA column, the levels of His-IL-37a protein in each of the samples from the loading samples were unbound and washed out and elution fractions in the purification procession were further checked by SDS-PAGE. As showed in Figure 3.4b, the IL-37 protein bands (about 22kDa) could be seen in both the soluble (loading sample) and insoluble fraction of bacterial lysis (lines 2 and 3), respectively. The proteins could also be detected in the flow though (unbound) fractions (lines 4 and 5); this could be due to the overloading of the samples beyond the binding ability of the Ni-NTA column. Importantly, most IL-37 proteins eluted from the column were in the fractions T7-T14 within the protein elution peak (Figure 3.4a). The IL-37a proteins were markedly purified compared with the loading sample (line 2) and unbound samples (line 4 and 5) using only the Ni-NTA column. Therefore, the Ni-NTA affinity purification is effective and successful in the initial purification of His-IL-37a proteins from *E. coli*.

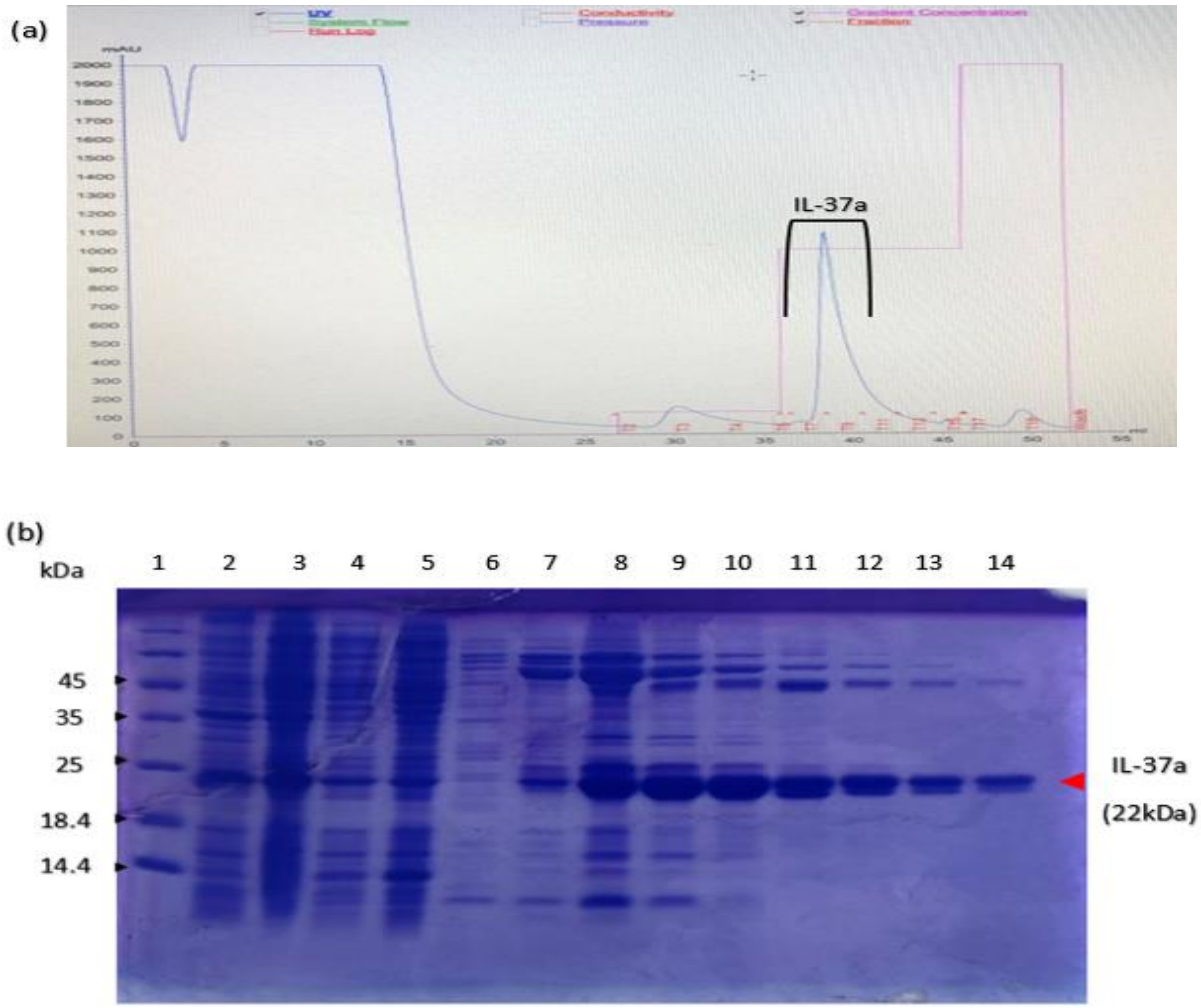



**Figure 3.4 Nickel affinity chromatography for IL-37a purification**

His-IL-37a protein was purified by 5ml His-Trap column using the AKTA protein purification system. a) The chromatography of recombinant IL-37a with Ni-NTA column. 35ml of soluble protein from total bacteria lysate were loaded onto the column, followed by washing with the elution buffer to wash away the unbound proteins (the flow rate was 2ml/min). The protein concentration was monitored using spectrophotometer at absorbance UV 280 nm. The peak of predicted IL-37a expression is highlighted by black brackets. b) SDS-PAGE result. Line 1: the 100kDa protein marker; Line 2: the soluble fraction of bacteria lysis. Line 3: insoluble fraction of bacterial lysis. Line 4: the flow through (unbound proteins); Lines 5 and 6: the elution fractions of weak band proteins (T5-T6). Lines 7-14: the elution fractions of IL-37a proteins (T7-14). The samples were mixed with SDS-PAGE loading buffer, 95ºC heating for 10 minutes. The protein samples were separated by 15% SDS-PAGE and stained with Coomassie Blue buffer for 40 minutes then de-stained. The result is representative of three experiments.

### 3.2.2.4 Purification of IL-37b by Ni-NTA chromatography

I next purified the 6His-tagged IL-37b protein. Since the kinetic of IL-37b induction in *E. coli* is similar to IL-37a (Figure 3.3) and the proteins were also soluble expressed in *E. coli*, I used the same method and purifying strategy for IL-37b production. The soluble proteins from total bacteria lysate which contains IL-37b were loaded onto the Ni-NTA column, followed by washing the column with elution buffer to wash away the unbound proteins (the flow rate was 2ml/min). The protein concentration in the flow through fractions was monitored using spectrophotometer. As showed in Figure 3.5a, unbound proteins were effectively removal by the washing and the expected IL-37b proteins were eluted from the column at 63% of the elution buffer level 100-180min after adding the elution buffer (Figure 3.5a). The fractions within the protein peak from T5-T12 were collected as potential recombinant IL-37b protein fractions.

SDS-PAGE was used to further check the purity and efficiency of IL-37b protein purified by the Ni-NTA column. The existence of IL-37b protein in the protein samples was identified by a protein band, size about 25kDa in 15% SDS gel (Figure 3.5b). As shown in Figure 3.5b, both the insoluble and soluble fraction (line 2 and 3) of bacterial lysis contain the 25kDa bands, suggesting that the recombinant IL-37b could also be soluble in the *E. coli* host. The washed-out fractions (lines 4 and 5) contained less IL37b compared with the loading samples (line 3). The 8 collected samples (T6-T12) within the eluted protein peak in the Figure 3.5a showed clear IL-37b protein band with higher purity compared with the loading sample (line 3) and the washed fractions (lines 4 and 5). Thus, the recombinant IL-37b purification by Ni-NTA column was effective and successful.

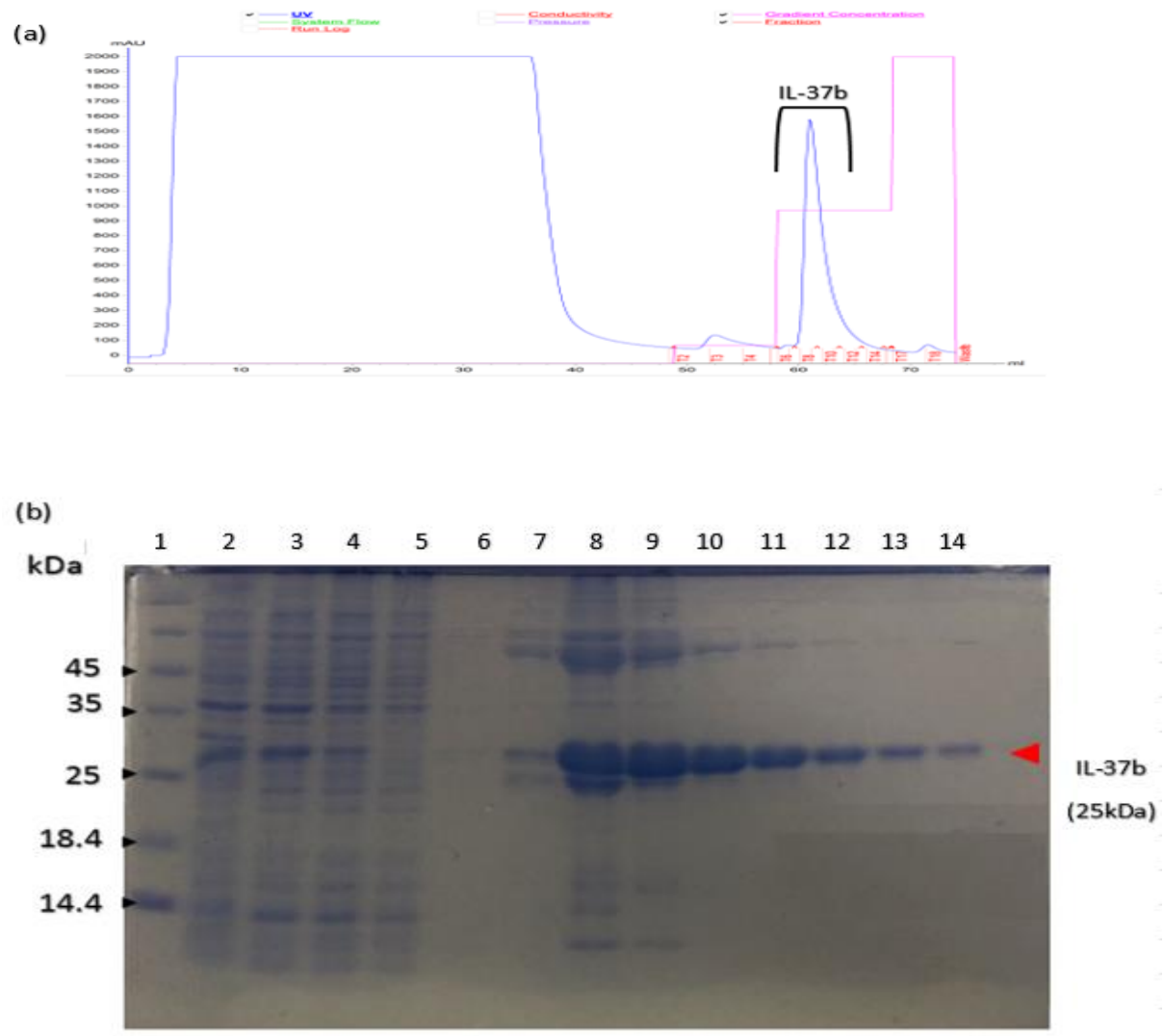



**Figure 3.5 Nickel affinity chromatography for IL-37b purification**

The His-IL-37b protein was purified by 5ml His-Trap column using the AKTA protein purification system. a) The chromatography of recombinant IL-37b using Ni-NTA column. 35ml of soluble protein from total bacteria lysate were loaded onto the column, followed by adding the elution buffer into the column to wash away the unbound proteins (the flow rate was 2ml/min). The protein concentration was monitored using spectrophotometer at absorbance UV 280nm. b) SDS-PAGE result. Line 1: the 100kDa protein marker; Line 2: insoluble fraction of bacterial lysis. Line 3: the soluble fraction of bacteria lysis. Line 4 and 5: the flow through (unbound proteins); Lines 6-12: the elution fractions of IL-37b proteins (T5-12). The samples collected from Ni-NTA elution were mixed with SDS-PAGE loading buffer, 95°C heated for 10 minutes. The protein samples were then separated by 15% SDS-PAGE and gel stained with Coomassie Blue for 40 minutes then de-stained. The result is representative of three experiments.

### 3.2.2.5 Confirmation of the His-tagged IL-37a and b by western blot

Although the SDS-PAGE results suggest that the proteins purified from the bacterial lysis were the IL-37a and b protein based on the molecular weight of the protein bands in the samples, it is essential to further confirm the recombinant proteins by western blot. Since there is no commercial anti-IL-37 antibody available for western blot so far, I used anti-6His-tag monoclonal antibody for the identification of the His-IL-37 recombinant protein. Briefly, the different protein samples in Figures 3.4 and 3.5 were collected before and after the Ni-NTA affinity purification. The protein samples were separated by SDS-PAGE and transferred onto Nylon membrane. The His-IL-37 proteins were identified with the conjugated anti- 6His-tag monoclonal antibody by western blot.

From Figure 3.6a, it was clearly observed that a single band about 20kDa existed in the bacterial lysate and eluted fractions but not in the washed-out fractions. The similar result was also obtained in IL-37b samples (Figure 3.6b). A single band around 25kDa was observed in all the samples by western blot. These results are consistent with the result in Figures 3.4 and 3.5 and confirmed that the 20 and 25 kDa protein bands in the samples are the His-IL-37a and His-IL-37b, respectively.

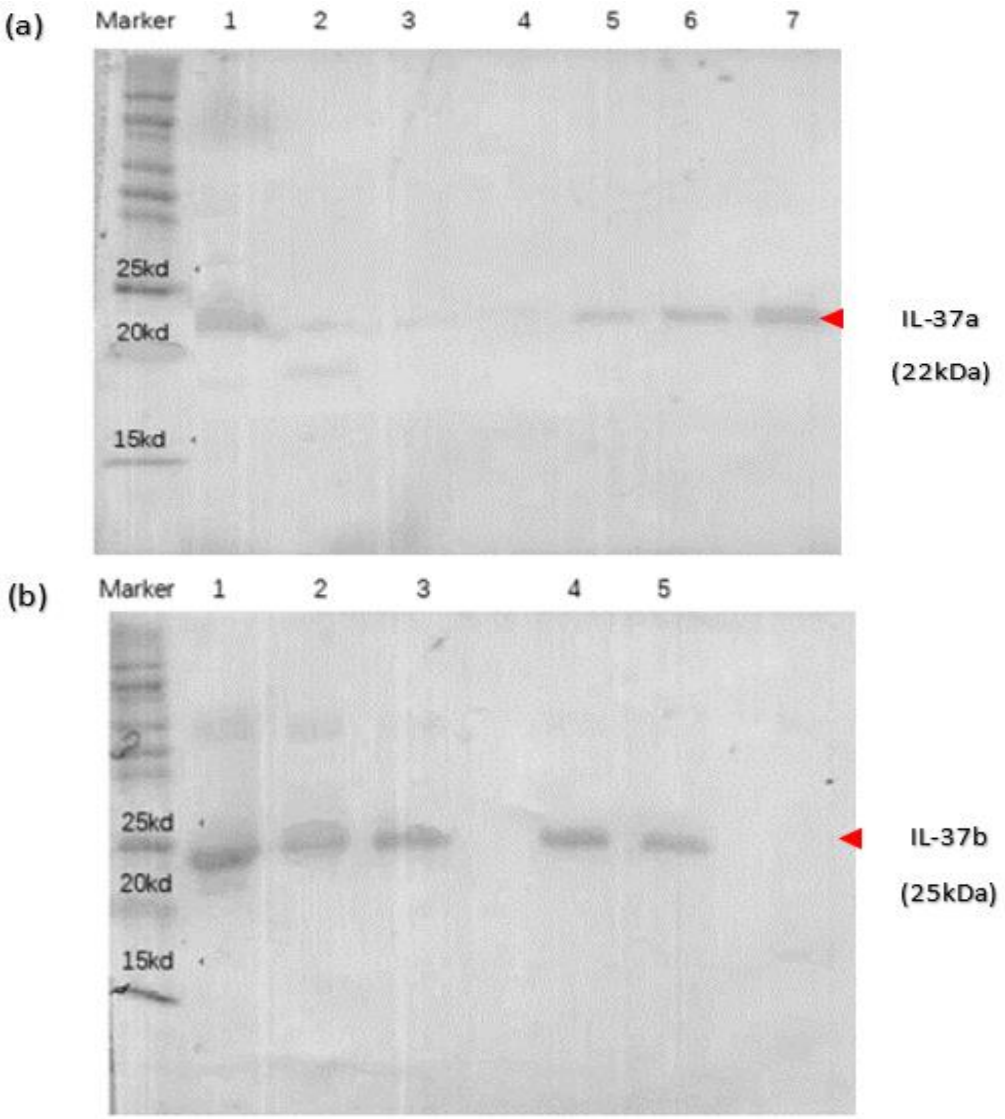



**Figure 3.6 The identification of His-tagged IL-37a and b proteins by western blot**

The recombinant His-IL-37 proteins were detected by western blot before and after protein purification. a) Western blot result of recombinant IL-37a. The first line was the protein marker (kDa); Line 1 was the soluble fraction of bacterial lysis, the loading sample; Lines 2-4 represent the washed-out proteins from the column. Lines 4-7 were the 4 eluted samples from Ni-NTA column. b) Western blot result of recombinant IL-37b. The first line was the protein marker (kDa); Line 1 was the soluble fraction of bacterial lysis, the loading sample; Lines 2,3 represent the washed-out proteins from the column. Lines 4,5 were the eluted samples from Ni-NTA column. The result is representative of three experiments.

### 3.2.2.6 Purification of IL-37a and b proteins by gel filtration

Although the Ni-NTA affinity chromatography was effective in the separation of His-IL-37 proteins from the whole bacteria proteins, several contaminated bacteria proteins could not be removed from IL-37 protein by this method. Therefore, the IL-37 proteins purified by Ni-NTA column needed to be further purified by size exclusion chromatography (SEC). This system separates proteins based on their difference in molecular weight (Figure 3.7a).

The recombinant IL-37a protein samples were loaded into the Superdex-75 SEC column equilibrated with DPBS buffer; the native condition maintains the protein bioactivity proteins. The proteins were separated with 1.5ml/min flow rate in cool room. Fractions (A11-B14) from the predicted protein peak which appeared 120min after sample loading were collected (Figure 3.7a). The purity of the IL-37a sample was checked by SDS-PAGE. Nearly all proteins in the sample were recombinant IL-37a, about 22kDa in size (Figure 3.7b).

Using the same method, the recombinant IL-37b protein was also further purified by gel filtration under the same purification condition. 115min after loading, a clear protein peak of recombinant IL-37b was observed (Figure 3.8a). All samples (A12-B8) from this peak were collected and the protein purity and size checked by SDS-PAGE gel (Figure 3.8b). On the SDS gel, sample B13 and B10 showed that the majority of protein bands were recombinant IL-37b, about 25kDa in size.

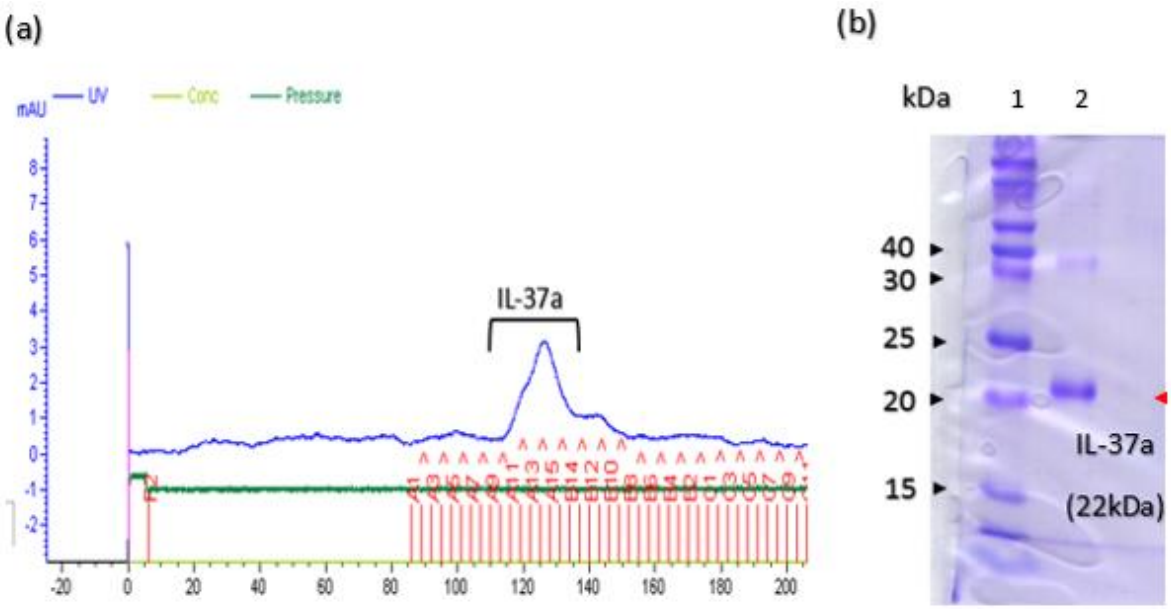



**Figure 3.7 Gel filtration purification of recombinant IL-37a proteins**

After Ni-NTA column purification, the recombinant IL-37a proteins were further purified by AKAT Pure gel filtration system.

a) Chromatograph of recombinant IL-37a with the Superdex-75 column of AKAT Pure system; the buffer flow rate was 1.5 ml/minute. The peak of interest protein was highlighted by black bracket.

b) The purity of the collected fraction after gel filtration was further checked by 15% SDS-PAGE gel with Coomassie blue staining and de-stained. Line 1 is the protein marker, line 2 is fraction A14 collected from gel filtration.

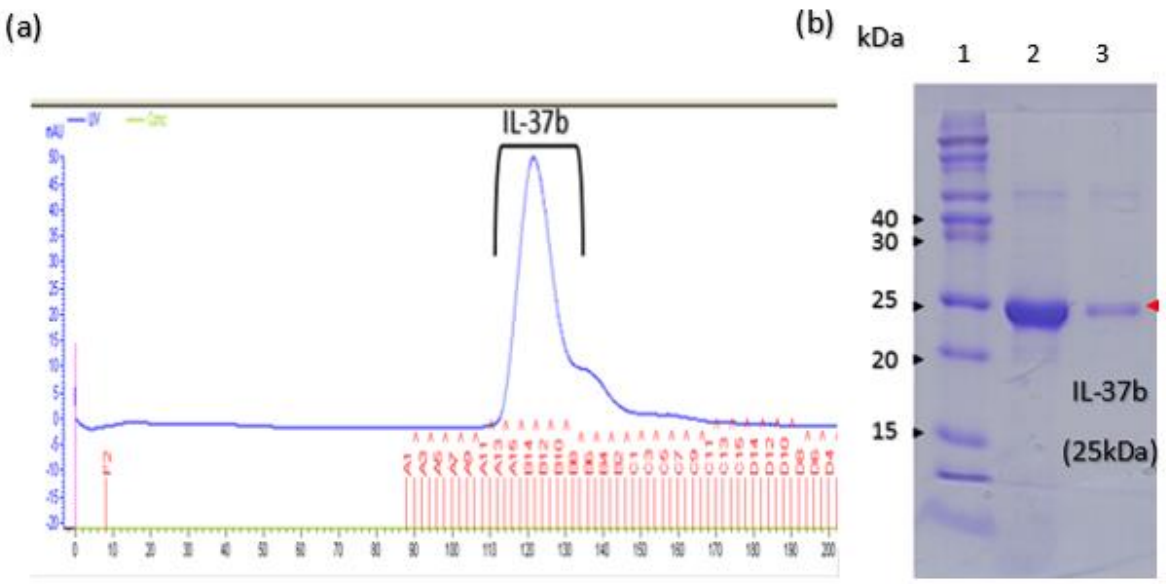



**Figure 3.8 Gel filtration purification of recombinant IL-37b protein**

The recombinant IL-37b protein from Ni-NTA purification was further purified by AKAT Pure gel filtration system.

a) Recombinant IL-37b sample was further purified using Superdex-75 column of AKAT Pure system; the filtration buffer flow rate was 1.5ml/minute. The peak of protein interested is highlighted by black bracket.

b) The fractions from gel filtration were further checked for purity by 15% SDS-PAGE gel with Coomassie blue staining and de-stained. Line 1 is the protein marker; line 2 and 3 were the protein fractions B13 and B10 collected from gel filtration.

### 3.2.2.7 Dialysis of purified IL-37a and b proteins

Dialysis is a commonly used method in protein purification. This method is mainly used as one of the purification steps to replace the protein solution with a suitable buffer which is better for protein stability and also for the next step of protein purification procedure (Macedo and Cordeiro, 2017). For instance, the imidazole in the elution buffer of Ni-NTA purification can be toxic to the cells in the subsequent bioassay of purified recombinant IL-37, therefore it must be removed by dialysis. Since the defined size of holes in the dialysis tube membrane allows only smaller molecules to pass and keeps the large molecules inside (Bollag and Edelstein, 1991) dialysis also allows to remove the contaminated small proteins.

An important issue in the dialysis is to ensure the protein stability and solubility during the buffer replacement via dialysis. Several physical and chemical factors may affect protein stability, such as protein nature, concentration, temperature, buffer pH, ionic strength and composition (Pesarrodona *et al.,* 2015). Among these factors, the buffer composition is the most important factor. Several buffers are commonly used for protein storage as well as dialysis, for instance, Tris–HCl, PBS, sodium phosphate and sodium citrate (Pesarrodona *et al.,* 2015) In this project, based on the published results and the protein features of IL-37 isoforms, I tested four dialysis buffers: Tris buffer, two different concentrations of NaCl with Tris and PBS in IL-37 protein purification process (Table 3.3). Both IL-37a and b proteins were stable, no precipitations had been seen in the buffer 1 which contained low concentration of NaCl (250mM) and Tris. IL-37a proteins were insoluble and precipitated in the other 3 buffers during the dialysis, while IL-37b proteins were also soluble in the buffer 2. The dialysis in buffer 1 had also the highest recovery, 86% for IL-37a and 90% for IL-37b, respectively. Thus, the buffer 1 was selected for the dialysis of both IL-37 protein isoforms.

| | Dialysis conditions | IL-37a concentration | | dialysis efficiency | Precipitation |
|---|---|---|---|---|---|
| | | before dialysis | after dialysis | | |
| 1 | 250 mM NaCl+ Tris 50 mM (PH=7.5) | 1.62mg/ml | 1.39mg/ml | 86% | — |
| 2 | 500 mM NaCl+ Tris 50mM (PH=7.5) | 1.62mg/ml | 1.32mg/ml | 82% | + |
| 3 | PBS (137 mM NaCl; 2.7 mM KCl; 8 mM Na2HPO4; 2 mM KH2PO4, pH=7.5) | 1.62mg/ml | 1.28mg/ml | 79% | + |
| 4 | Tris buffer (PH=7.5) | 1.62mg/ml | 1.24mg/ml | 76.50% | + |

| | Dialysis conditions | IL-37b concentration | | dialysis efficiency | Precipitation |
|---|---|---|---|---|---|
| | | before dialysis | after dialysis | | |
| 1 | 250 mM NaCl+ Tris 50 mM (PH=7.5) | 2.21mg/ml | 1.99mg/ml | 90% | — |
| 2 | 500 mM NaCl+ Tris 50mM (PH=7.5) | 2.21mg/ml | 1.87mg/ml | 85% | — |
| 3 | PBS (137 mM NaCl; 2.7 mM KCl; 8 mM Na2HPO4; 2 mM KH2PO4, pH=7.5) | 2.21mg/ml | 1.83mg/ml | 83% | + |
| 4 | Tris buffer (PH=7.5) | 2.21mg/ml | 1.24mg/ml | 77.00% | + |

**Table 3.3 Different buffers for IL-37a and b protein dialysis and stability**

### 3.2.2.8 Endotoxin removal of recombinant IL-37a and b proteins

I sought next to remove the endotoxin from IL-37 proteins produced in *E. coli* by polymyxin B columns. The Polymyxin B Sepharose gel column can bind micrograms of endotoxin so that it is an effective method to remove LPS from the recombinant IL-37 proteins produced in *E. coli*. The endotoxin levels in the recombinant proteins were measured by Limulus Amebocyte lysate (LAL) QCL-1000 pyrogen test kit (Mølvig and Back, 1987), before and after endotoxin removal. The endotoxin levels in IL-37a and b recombinant proteins were 0.0235 EU/μg and 0.0357EU/μg, respectively, before the polymyxine B purification. After endotoxin removal, the endotoxin levels in recombinant IL-37a and b were less than 0.01EU/μg (0.00483EU/μg and 0.00957EU/μg, respectively), which is an acceptable level of endotoxin for cell culture and animal experiment.

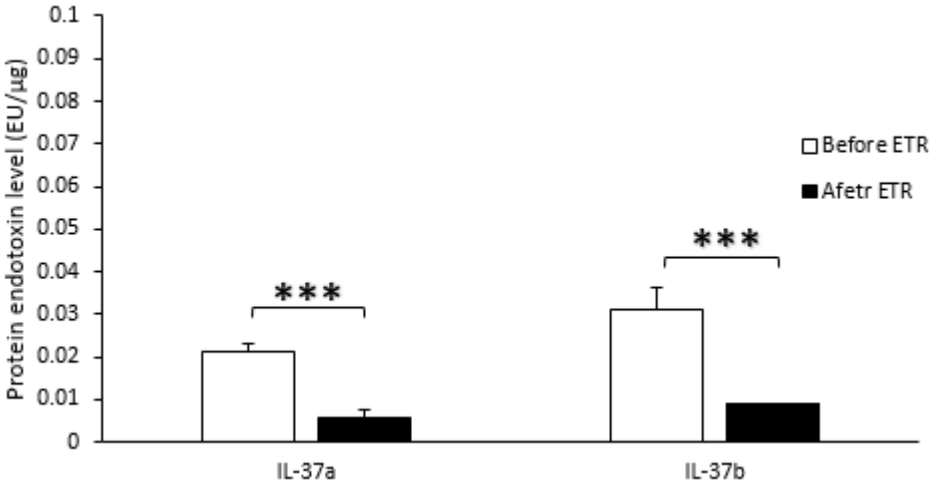



**Figure 3.9 Comparison of the endotoxin level in IL-37a and IL-37b protein products before and after endotoxin removal**

Endotoxin in the recombinant IL-37a and b was removed by polymyxin B affinity purification according to the instruction of manufacturer. The endotoxin levels in the two samples were detected by endotoxin detection kit (ETR= endotoxin removal). The endotoxin levels of IL-37a/b after ETR are compared with the level before ETR. Using student T-test, data are means ± SD and are representative of three independent measurements. ***P<0.001 compared to control.

### 3.2.3 Production of IL-37a and b monomer proteins

Following the recent report that the dimerization of IL-37b proteins can dampen their bioactivity and immune suppress function (Ellisdon *et al.,* 2017), it is important to produce the monomeric IL-37b protein for research. Furthermore, it is currently unknown if IL-37a can also form homodimer and whether this can also affect its function. In this part of the project, I firstly determine the dimerization possibility of IL-37a protein by electronic remodelling, then design the mutant IL-37a and b gene for the expression and production of monomeric IL-37a and b proteins in *E. coli*, respectively.

#### 3.2.3.1 Determine the dimerization possibility of IL-37a

Based on IL-37b protein structure, the IL-37a protein structure can be predicted with SWISS-MODEL using the comparative modelling engine (Waterhouse *et al.,* 2018). As with the previously published result that IL-37b proteins can form head to head homodimers via their Try85 (Ellisdon *et al.,* 2017, Figure 3.10b), I found that IL-37a can also form homodimer in the similar way, but via Try59 (Figure 3.10a). Intriguingly, IL-37a and b may also form heterodimer via their Try within each of the two isoform proteins (Figure 3.10c).

#### 3.2.3.2 Plasmid construction for the expression of IL-37a and b protein monomer in *E. coli*

After identifying the amino acid (tyrosine) responsible for the dimer formation of IL-37a and b, I next replaced the tyrosine (Y) with alanine (A) by point mutation in IL-37a and b cDNA. This has successfully prevented IL-37b dimerization (Ellisdon *et al.,* 2017). Briefly, the DNA sequence $TAC^{175}$ (encoding Y59) in IL-37a cDNA and $TAC^{294}$ (encoding Y85) in IL-37b cDNA were changed into GCC (encoding Alanine) for monomeric IL-37a and b production, respectively in *E. coli*. The cDNA sequences for encoding mutant IL-37a and b proteins were synthesised and inserted into the expression vectors PET-21a and PET-28a (Figure 3.11) and transformed into host *E. coli*. The sequence confirmed plasmids for the expression of mutant IL-37a (Y59A) and IL-37b (Y85A) were used for the monomeric protein production as above.

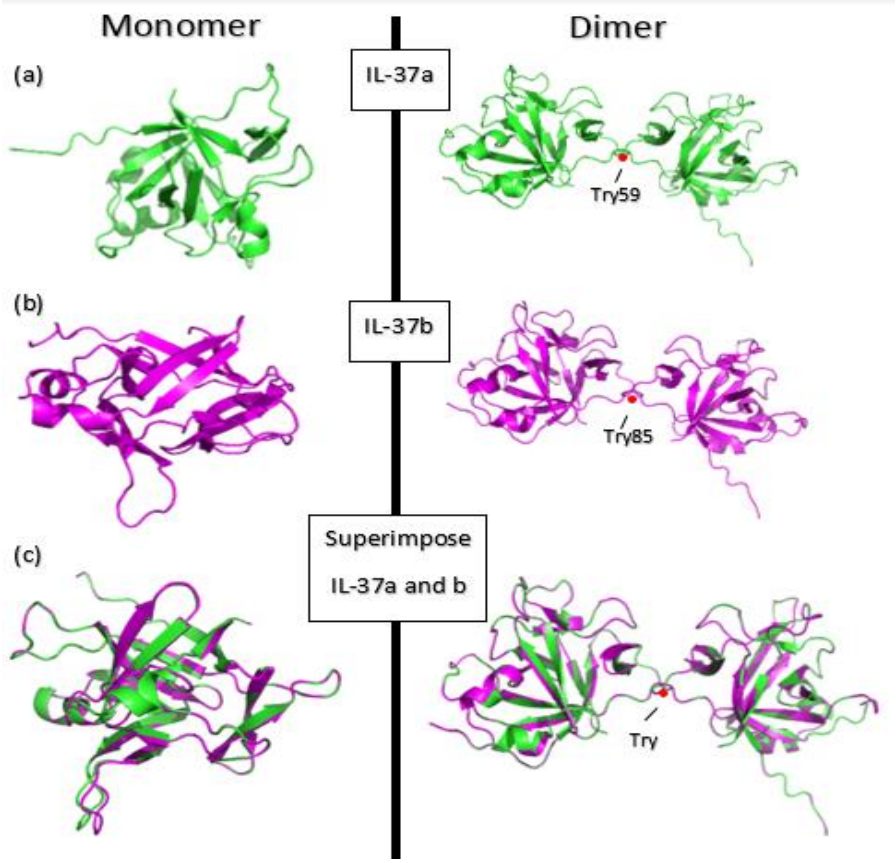



**Figure 3.10 The difference in monomer and dimer formation between IL-37a and IL-37b proteins**

The electronic modelling was performed by the software SWISS-MODEL and Phyre2. The dimer forming site marked by red point.

a) The modelling of monomer and homodimer structure of IL-37a protein (Green).

b) The modelling result for monomer and homodimer structure of IL-37b protein (Purple).

c) Superimposing of monomer and heterodimer IL-37a and b protein.

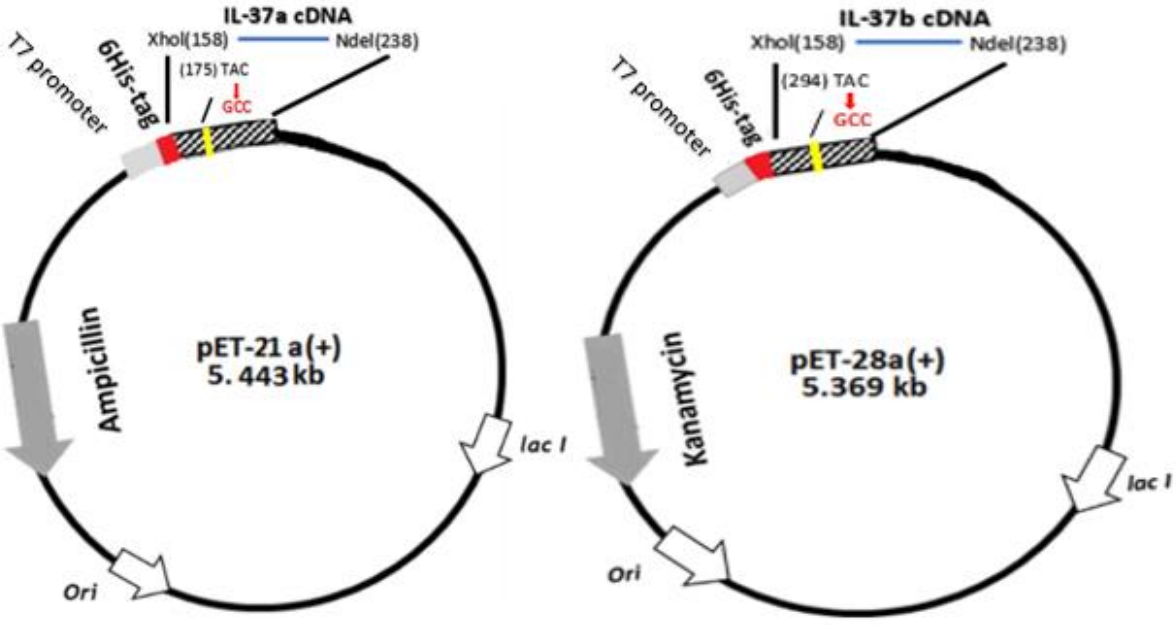



**Figure 3.11 Schematic representation of plasmid DNA for the expression of mutant IL-37a and b monomeric protein in *E. coli*** (changing the cDNAs to IL-37a (Y59A) and IL-37b (Y85A), also changing the antibiotic resistant genes).

The PET-21a and PET-28a vectors were used to express mutant IL-37a (Y59A) and IL-37b (Y85A) proteins. The mutant IL-37a and b cDNA sequences were synthesised and inserted between the Xho I and Nde I sites in the two plasmids, respectively. Ori means the origin of replication; LacI is the LacI coding sequence; dark grey areas are the antibiotic resistant gene coding sequence (Ampicillin resistant gene for PET-21a, Kanamycin one for PET-28a); light grey areas are T7 promoters; the red areas are the coding sequences of 6His Tag.

### 3.2.3.3 Nickel affinity chromatography for mutant IL-37a and b purification

The mutated IL-37a and b proteins were purified using the same strategy and method as the natural form IL-37 proteins. His-tagged IL-37 mutants bound to the Ni-NTA column and eluted by the increasing dose of imidazole. Figure 3.12a showed the Nickel affinity purification of IL-37a Y59A mutant proteins from *E. coli* lysate. Figure 3.13a shows the result of IL-37b Y85A mutant purification by Nickel chromatography. The presence and purity of IL-37a and b mutants after Nickel purification were confirmed by SDS-PAGE (Figure 3.12b and Figure 3.13b). The results demonstrated that the mutant IL-37a and b proteins could also be purified by the same method as the native ones.

### 3.2.3.4 Purification of mutant monomeric IL-37a by gel filtration

Since the mutant IL-37 proteins purified by Nickel affinity chromatography N were still contaminated with bacterial proteins, it was necessary to further purify the proteins by size exclusion chromatography (SEC). The mutant IL-37a proteins collected from Nickel purification were further separated in Superdex-75 column of AKTA pure system. Three protein fractions eluted in the predict protein peak were collected (Figure 3.14a). The existence and purity of the IL-37a proteins were confirmed by SDS-PAGE gel (Figure 3.14b). Thus, the mutant IL-37a proteins were successfully produced and purified.

The mutant IL-37b proteins were also produced and purified in the same way successfully (Ellisdon *et al.*, 2017, data not shown).

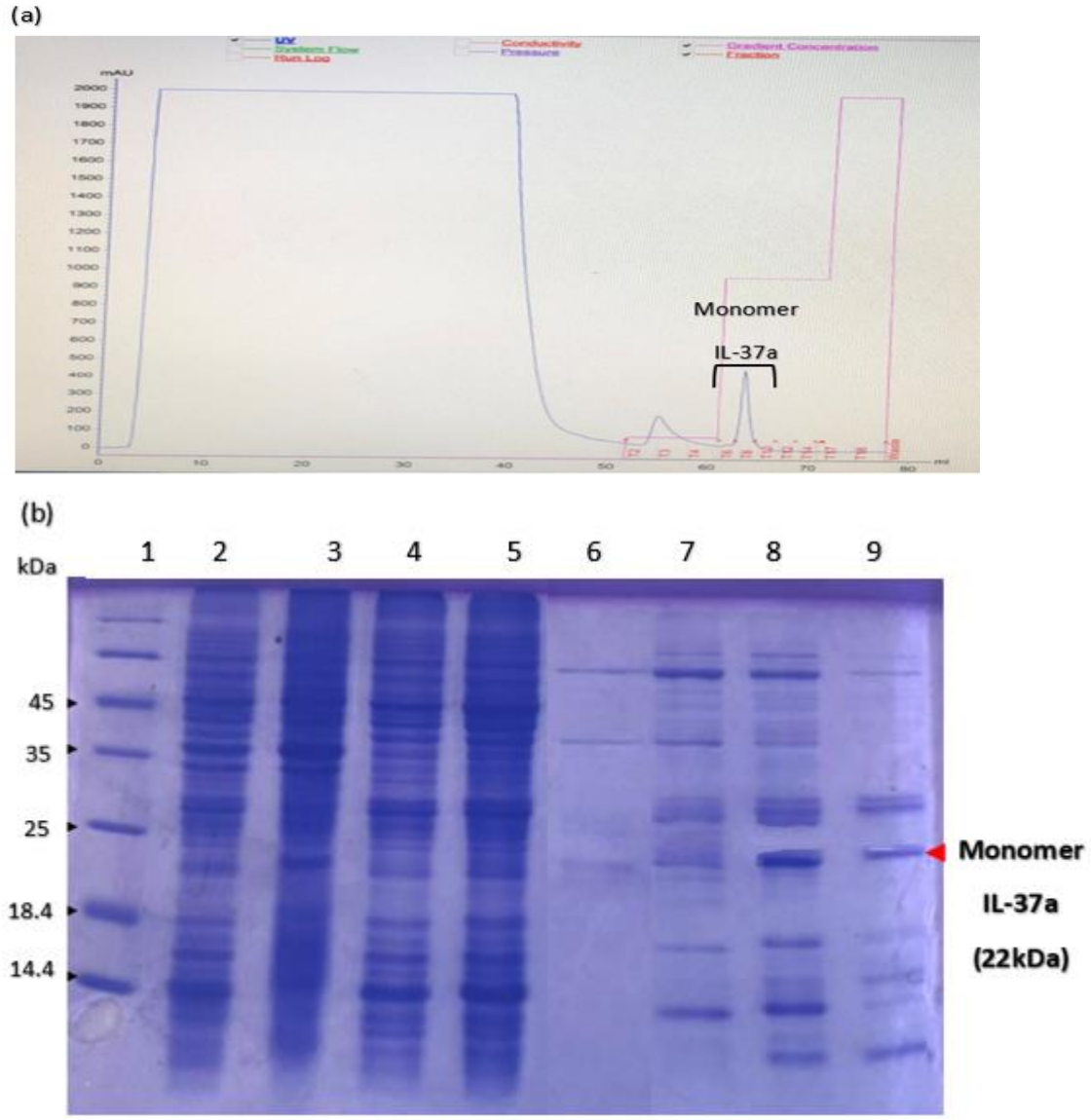



**Figure 3.12 Nickel affinity chromatography for mutant IL-37a purification**

The mutant 6His-IL-37a proteins were purified by His-Trap column using the AKTA protein purification system. a) The chromatography of IL-37a mutant with Ni-NTA column, flow rate was 2ml/min; the peak of predicted IL-37a mutant proteins is highlighted by black brackets and protein fractions were collected. b) SDS-PAGE result. 5 fractions collected from Nickel chromatography were separated by 15% SDS- gel. Line 1: the 100kDa protein marker; Lines 2 and 3: the soluble loading protein and insoluble bacteria lysate. Line 4-6: the unbound flow-through proteins; Lines numbered 7-11: were the eluted fractions of mutant IL-37a by different concentration of imidazole.

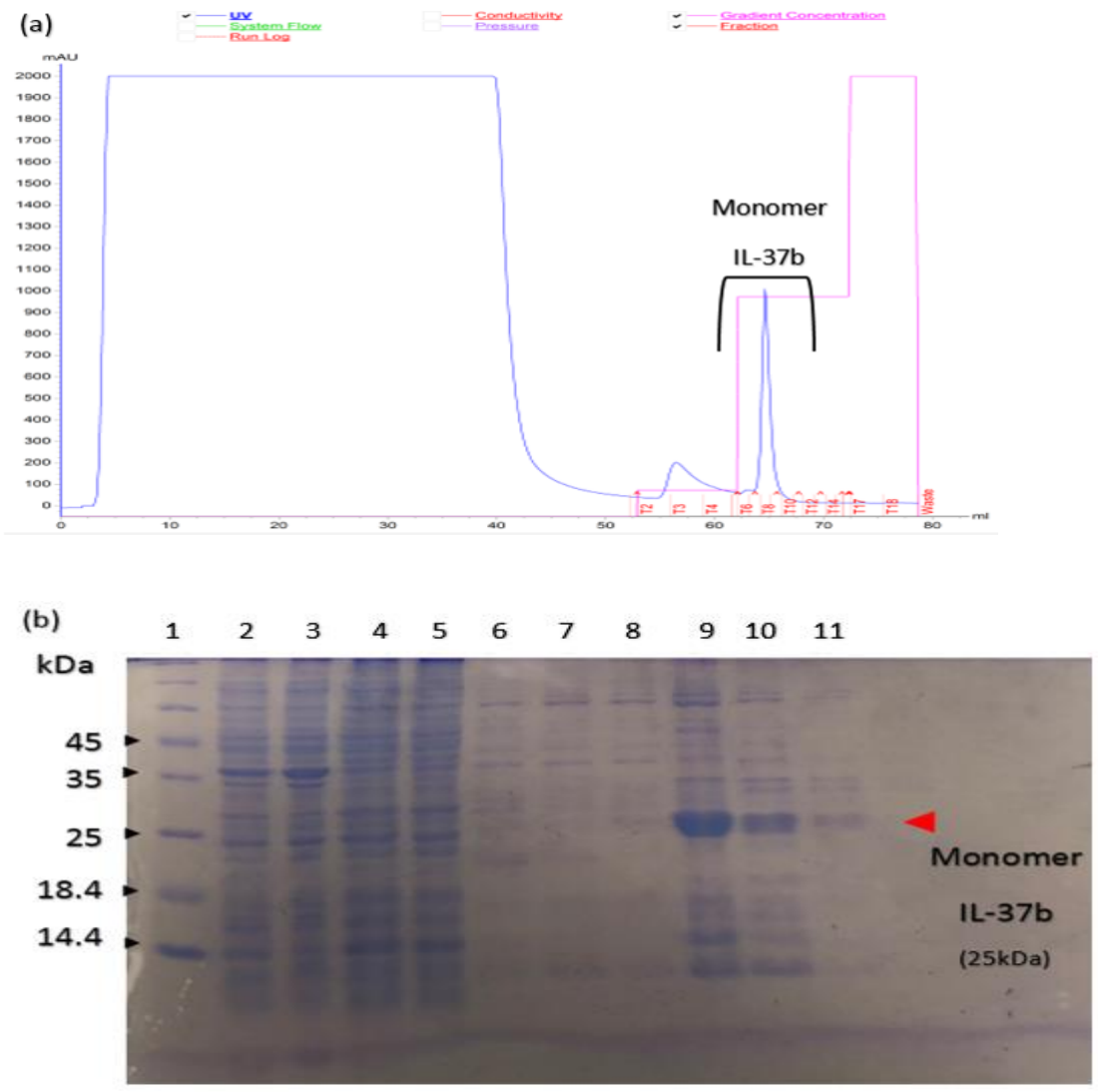



**Figure 3.13 Nickel affinity chromatography for monomer IL-37b purification**

Mutant His-IL-37b protein was purified by His-Trap column using the AKTA protein purification system. a) The chromatography of mutant IL-37b with Ni-NTA column, flow rate was 2ml/min; the protein peak of predicated IL-37b elution was highlighted by black brackets. b) SDS-PAGE result.

5 fractions collected from Nickel chromatography were separated by 15% SDS-PAGE gel. Line 1: the 100kDa protein marker; Lines 2 and 3: the loading soluble loading protein and insoluble bacteria lysate. Line 4-6: the unbound flow through proteins; Lines 7-10: the eluted fractions of mutant IL-37b by increasing concentration of imidazole.

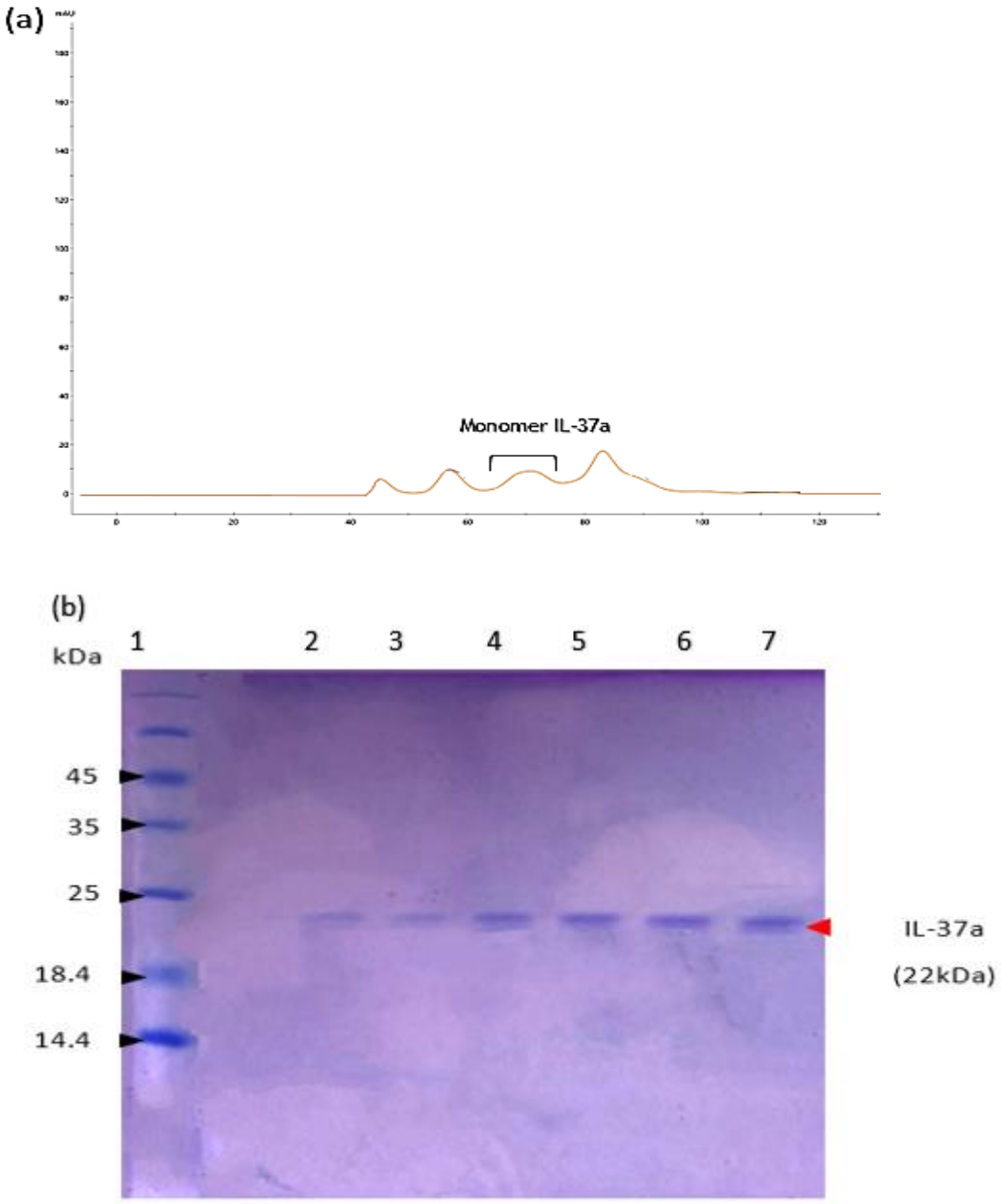



**Figure 3.14 Purification of mutant IL-37a proteins by gel filtration**

The recombinant IL-37a mutant proteins from Ni-NTA column were further purified by gel filtration. a) Recombinant IL-37a monomer (orange line) information in Superdex-75 column of AKAT Pure system; with filtration buffer flow rate of 1.5ml/minute. The peak of interest protein highlighted by black bracket. b) Collected protein fractions from gel filtration were further checked by 15% SDS-PAGE gel with Coomassie blue staining and de-stained. Line 1: 100kDa protein ladder; lines 2-7: 6 fractions collected from gel filtration.

### 3.2.3.5 Identification of the monomer and dimer protein of recombinant IL-37a and b by native PAGE

We next determined whether the mutant IL-37a and b proteins that we produced are in monomeric form. Whereas the SDS-PAGE is useful to estimate the molecular weight of protein, it cannot be used to identify the IL-37 dimers. This is because the mobility of a protein in a PAGE is determined by both charge and size and SDS can disrupt the protein structure to produce a linear polypeptide chain coated with negatively charged SDS molecules. Therefore, the protein mobility in SDS PAGE depends mainly on its molecular weight. In contrast, in the native PAGE, proteins are prepared and run without detergent, which maintains both the proteins' secondary structure and native charge density, thus, the protein movement during the electrophoresis will mainly depend on their primary amino acid sequence which can reflect the real protein molecular weight (Arndt *et al.*, 2012). Therefore, the native polyacrylamide gel electrophoresis can be used to distinguish protein monomer and dimer, such as the native and mutant IL-37a and b proteins.

Three samples each of the natural and mutant (monomers) of IL-37a and b proteins, respectively were separated on the 15% native PAGE gel. The electrophoresis system was run in ice-cold condition to maintain the protein structure. It was expected that the monomer for IL-37a and b should be around 22 and 25kDa, respectively and the dimer over 40 and 50kDa, respectively. As shown in the Figure 3.15a, the natural IL-37a proteins (sample 4-6) consisted of both the monomer and dimers. In contrast, the mutant IL-37a proteins (samples 1-3) were only presented in the monomeric state. It was noted that there were also some bands with sizes in between the monomer and dimer IL-37, the nature of the proteins is unknown.

The similar result was also obtained with the natural and mutant IL-37b proteins (Figure 3.15b).

Thus, the result demonstrated that mutagenesis of IL-37a (Y59A) can prevent the dimerization of IL-37a. Our results also confirmed that mutation of IL-37b (Y85A) is sufficient to avoid IL-37b dimerization, as reported (Ellisdon *et al.*, 2017).

In summary, I have successfully produced both natural and mutant IL-37a and b proteins. As summarised in the Table 3.4, from 5 litres of bacterial culture, 9.4 mg of natural and 8.1mg of mutant IL-37a protein and 5.4mg of natural and 10mg of mutant IL-37b proteins have been produced. The recombinant proteins were highly purified by both Nickel affinity chromatography and gel filtration. The endotoxin levels in the purified protein samples were less than 0.01EU/μg in most of the protein preparations.

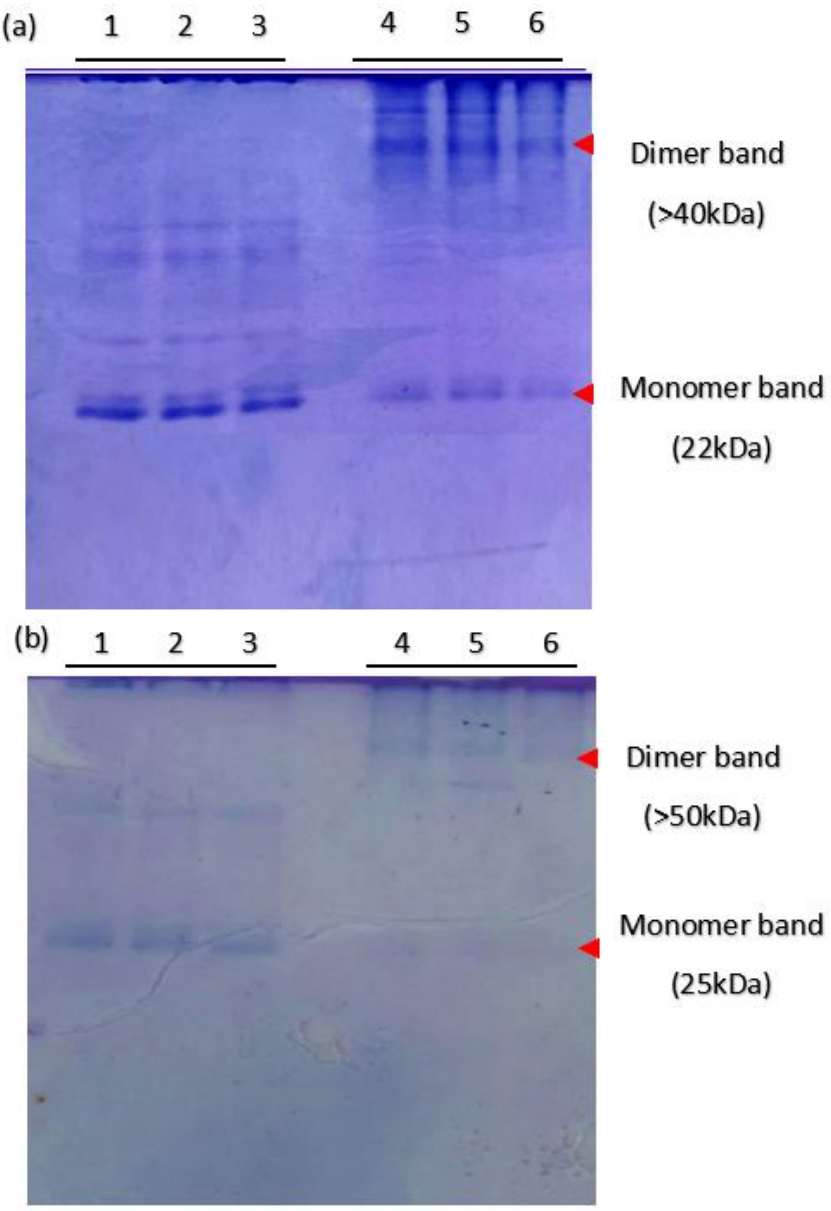



**Figure 3.15 Identification of IL-37a and b monomers and dimers by native PAGE**

The monomer and dimer state of both recombinant IL-37a and b proteins was confirmed using 15% native PAGE gel. a) Native PAGE result of natural and mutant IL-37a proteins. Line 1-3: 3 samples from mutant IL-37a (recognized as monomer) purified by gel filtration; Line 4-6: 3 samples from natural IL-37a purified by gel filtration. Predicted monomer size is about 22kDa, dimer size is >40kDa. b) Native PAGE of natural and mutant IL-37b proteins. Line 1-3 were the 3 samples of purified mutant IL-37b (recognized as monomer). Line 4-6 were the samples from natural purified IL-37b. Predicted monomer size is about 25kDa, dimer size is >50kDa.

| Protein | IL-37a | | IL-37b | |
|---|---|---|---|---|
| | natural | mutant | natural | mutant |
| **Optimized induction time** | 5h | 4h | 5h | 4h |
| **Bacteria volume** | 5 litres | 5 litres | 5 litres | 5 litres |
| **Bacteria weight** | 25.2g | 30.5g | 31g | 33g |
| **Dialysis efficiency** | 89.20% | 86% | 83.50% | 85.70% |
| **Protein concentration** (by Coomassie blue) | 0.650mg/ml | 0.752mg/ml | 0.320mg/ml | 1.116mg/ml |
| **Protein volume** | 12.5ml | 12.5ml | 17ml | 9ml |
| **Yield** | 8.13mg | 9.4mg | 5.44mg | 10.044mg |
| **Endotoxin level** | 0.008EU/μg | 0.0074EU/μg | 0.01EU/μg | 0.0097EU/μg |

EU= endotoxin unit

**Table 3.4 Summary of the production and purification of wild type and mutant IL-37a and IL-37b proteins in *E. coli***

### 3.2.4 Generation of human IL-37a transgenic and receptor knockout mice

To investigate the role of human IL-37a in disease *in vivo*, we generated human IL-37a-transgenic (IL-37aTg) mice via a company (Taconic Biosciences GmbH). The IL-37 receptor IL-1R8/IL-1R8 knockout mice (IL-1R8KO) were kindly obtained from Professor Alberto Mantovani (Garlanda *et al.*, 2004).

All mice were housed in specific–pathogen-free conditions at the Biological Service, University of Glasgow. 6-10 week-old mice were used in the experiments under licence and in accordance with the UK Home Office guidelines.

#### 3.2.4.1 Generation and identification of IL-37a transgenic mice

The transgenic mice were generated using the standard method (Xiang *et al.*, 2010). Briefly, full-length human IL37a cDNA (NM_173205.1) was inserted downstream of the CAG promoter in the vector pIRES (Figure 3.16a). The cloning plasmids were linearized by endonuclease digestion and the gel-purified DNA fragments were injected into the pronucleus of fertilized zygotes harvested from C57BL/6 mice, using conventional microinjection methods for the generation of transgenic mice (Nold *et al.*, 2010).

Two pairs of PCR primers were designed to identify the transgenic line (Figures 3.16b and c). The primer pair A was designed for the amplification of a 379bp fragment between CAG promoter and the 5'-end of IL-37a cDNA with oligo1 and 2 (Figure 3.16b); the primer pair B was designed for the 401bp fragment amplified with oligo3 and 4 between the 3'-end of IL-37a cDNA and hGHpa site on the plasmid (Figure 3.16c). If the transgenic mouse genome carries both the 379bp and the 401bp fragment detected by PCR, the mouse can be identified as IL-37a transgenic mice. If the mouse genome carries only one of the fragments, this suggest that the mouse carries a truncated IL-37a transgenic construct. Thus, with the two primer pairs, IL-37a transgenic line (IL-37aTg) can be successfully detected by PCR.

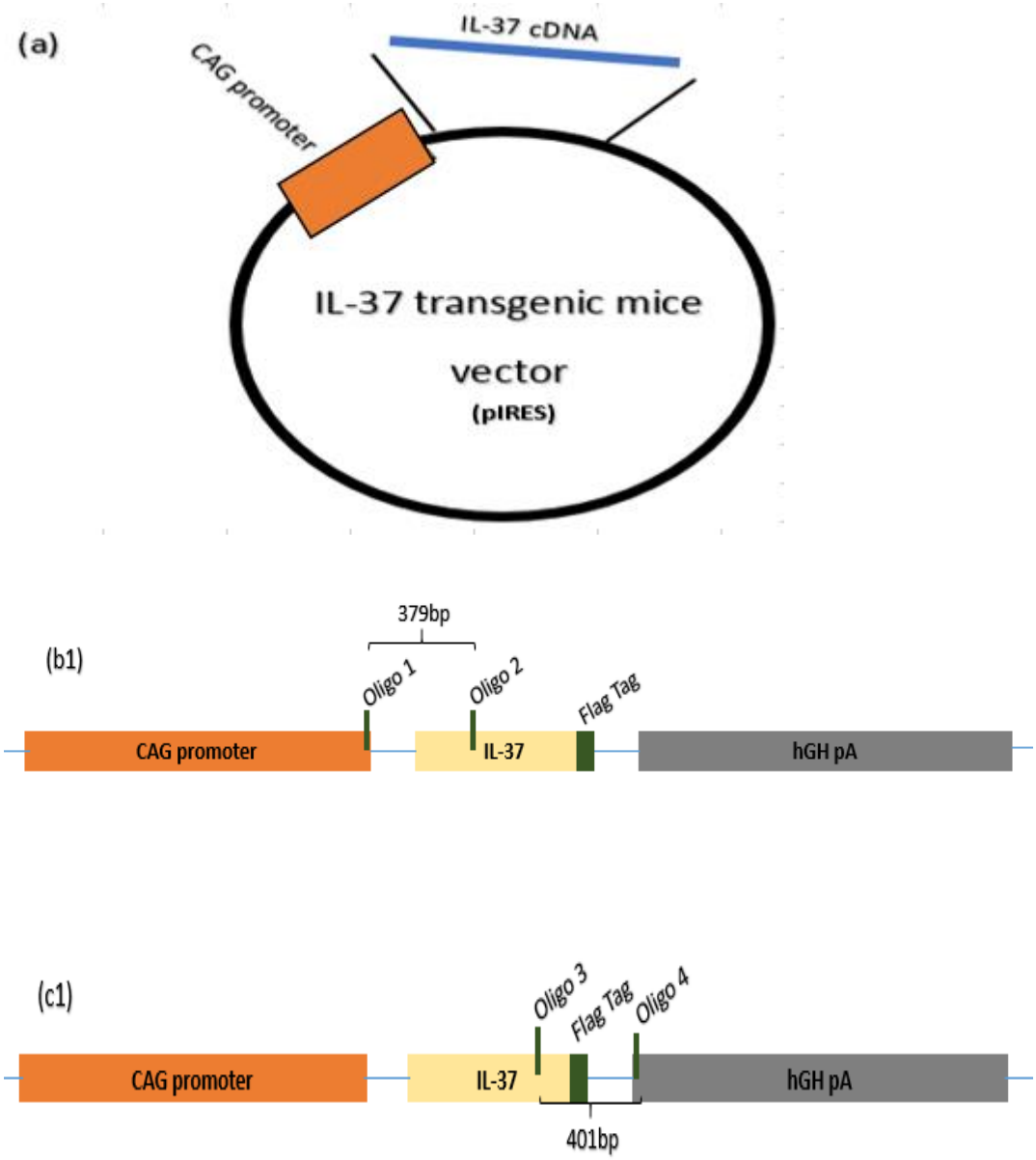



**Figure 3.16 The transgene construct and the identification of human IL-37 transgenic mice by PCR**

a) Schematic representation of Transgenic IL-37 construct.

b) The PCR primer pair A for the identification of 3' -terminal of IL-37a transgene construct.

c) The location of PCR primer pair B for the identification of 5' of IL-37 transgene construct.

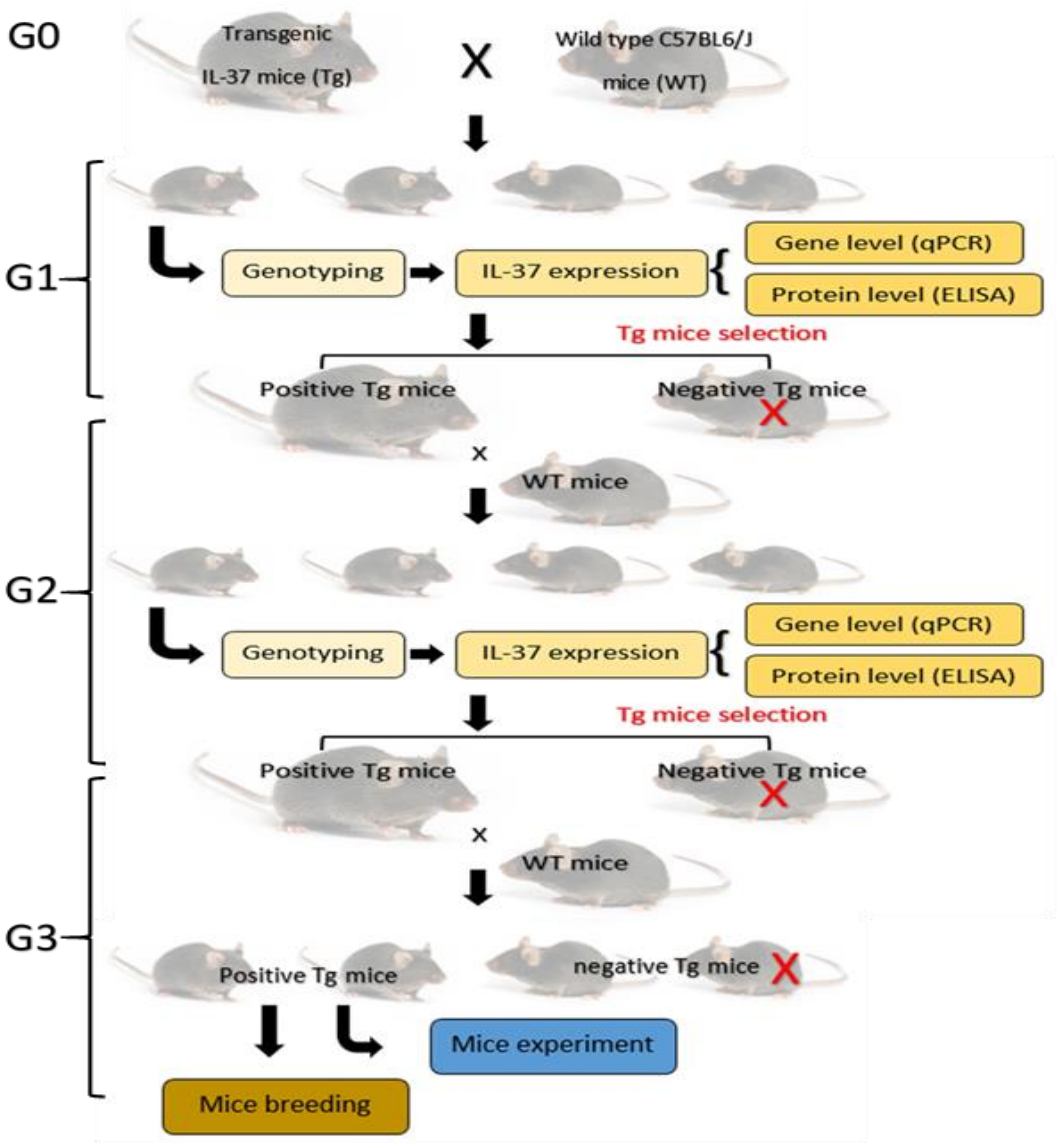



**Figure 3.17 Transgenic mice crossbreeding process flow chart**

The founder IL-37aTg mice (G0) were mated with WT mice for 3 generations. The transgenic mice were identified by genotyping with PCR using specific primers. The expression level of IL-37 mRNA and protein in each generation were measured by qPCR and ELISA. The stable transgenic mice were kept for further breeding and animal experiments.

### 3.2.4.2 The breeding and identification of IL-37aTg mice

Since the transgenic IL-37a mouse lines that we received were the F0 founder animals, the lines have to be further bred for at least three generations in order to identify the stable transgenic mice for experiment and breeding. This is because the transgenic construct is randomly inserted into the host genome so that its stability and the level of transgene expression can vary in different founder mice. Thus, it is important to screen the transgenic lines in that the transgene is stably inherited and has appropriate expression level of transgene. Since the IL37aTg mice are in C57BL/6 background the same background WT mice were used to cross with the Tg mice. The strategy for the breeding of IL37aTg mice was illustrated in Figure 3.17. The stability and expression level of IL-37a transgene in each generation of the IL37aTg mice were determined and compared. All the breeding was carried out in VRF (Veterinary Research Facility), University of Glasgow. The confirmed F3 IL-37aTg mice were kept for further breeding and experiments.

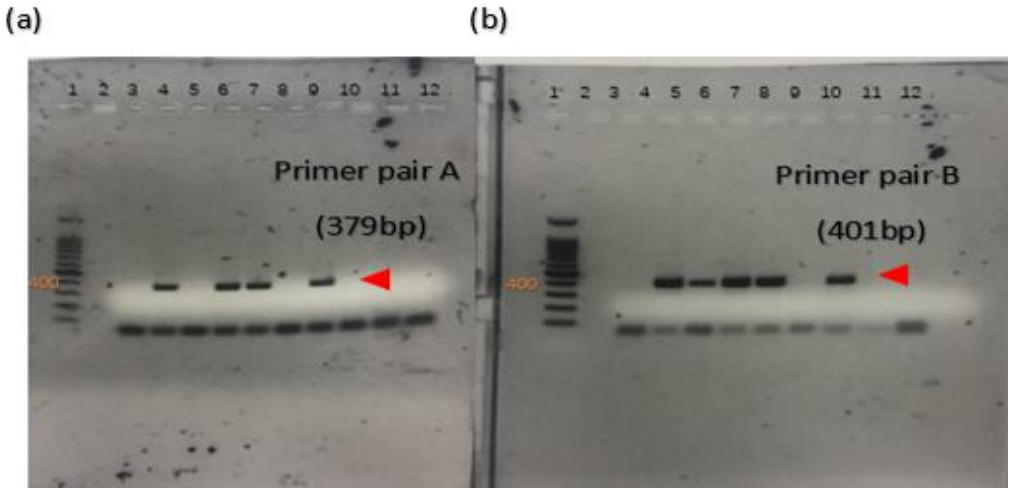



**Figure 3.18 Genotyping identification of IL-37a transgenic mice by PCR**

Mice ears were lysate and extract DNA from them, followed by two specific primer pairs PCR reaction. The PCR result were checked by 1.5% agarose gel.

a) IL-37aTg mice detected by primer pair A. line 1: 100bp DNA marker; Line 3-11: test sample from 9 transgenic mice litters; Line 2,12: negative control. Predict IL37aTg band at 379bp.

b) IL-37aTg mice detected by primer pair B. line 1: 100bp DNA marker; Line 3-11: test sample from 9 transgenic mice litters; Line 2, 12: no DNA template control. Predict IL37atg band about 401bp.

The result was the representative of 80 genotyping results.

### 3.2.4.3 Genotype and stability of IL-37aTg mice

After weaning of each new litter of IL-37aTg mice, ear samples were collected by ear puncher (Project licence ID: PD13F11FC) and genomic DNA extracted for genotyping by PCR using both Primer pairs A and B as described before (Figure 3.16c). The Figure 3.18 is a representative of many genotyping results. 9 DNA samples from one litter of 9 mice produced by the IL37aTg and WT parents were extracted, and PCR performed for IL-37aTg construct. The specific IL-37aTg fragment (379bp) amplified by the primer pair A was detected in 4 of 9 samples (Figure 3.18a). The presence of specific IL-37aTg band (401bp) amplified by primer pair B was also observed in 5 of 9 samples (Figure 3.18b). Based on the criterion that only the Tg mouse whose DNA contains specific IL-37Tg bands amplified by both primer pairs A and B were considered to be the successful IL-37aTg mouse, the mouse number 4, 6 and 7 were the IL-37aTg mice in this experiment. The mouse DNA samples (number 5, 8 and 10) only produced one band either by primer pair A or B, and therefore were not successful transgene, this may be due to a partial insertion of the IL37a transgenic construct.

The genotyping method is also useful to determine the stability of transgene in mice. According to the principle of heredity, around 40% of the offspring from the transgenic and WT parents should carry the transgene successfully. In my genotyping results of IL-37aTg mice, the majority of the breeders had around 50% transgene rate for at least three generations (Table 3.5 and data not shown). Therefore, this strain of IL37aTg mice were breeding successfully and the transgene is stably inherited.

| ID | male | | female | male genotype | female genotype | mice number | positive mice | positive rate |
|---|---|---|---|---|---|---|---|---|
| 1 | 2096947 | x | VRF10136 | Tg | WT | 20 | 12 | 60.00% |
| 2 | VRF10137 | x | 2097165 | WT | Tg | 27 | 15 | 55.56% |
| 3 | VRF10137 | x | 2096964 | WT | Tg | 16 | 6 | 37.50% |
| 4 | VRF10137 | x | VRF9960 #5 | WT | Tg | 16 | 8 | 50.00% |
| 5 | VRF10137 | x | VRF9960 #7 | WT | Tg | 15 | 8 | 53.33% |
| 6 | VRF10137 | x | VRF10101 #15 | WT | Tg | 14 | 8 | 57.14% |
| 7 | VRF11001 B #22 | x | VRF 13679 C57 | Tg | WT | 20 | 13 | 65.00% |
| 8 | VRF11006 A #37 | x | VRF 13679 C57 | Tg | WT | 22 | 15 | 68.18% |
| 9 | VRF10113 A #27 | x | VRF 13679 C57 | Tg | WT | 15 | 7 | 46.67% |
| 10 | VRF12124 A #91 | x | VRF 13679 C57 | Tg | WT | 11 | 7 | 63.64% |
| 11 | VRF12124 A #91 | x | VRF 13679 C57 | Tg | WT | 12 | 7 | 58.33% |
| 12 | VRF13679 C57 | x | VRF12125 C #104 | WT | Tg | 13 | 7 | 53.85% |
| 13 | VRF10137 | x | VRF12947 B #172 | WT | Tg | 18 | 11 | 61.11% |
| 14 | VRF15164 #1 | x | VRF12947 B #173 | WT | Tg | 14 | 6 | 42.86% |
| 15 | VRF15164 #2 | x | VRF12947 C #174 | WT | Tg | 14 | 8 | 57.14% |
| 16 | VRF11001 B #22 | x | VRF12948 B #181 | Tg | Tg | 12 | 7 | 58.33% |
| 17 | VRF15164 #3 | x | VRF12948 B #180 | WT | Tg | 17 | 10 | 58.82% |
| 18 | VRF15164 #4 | x | VRF12947 C#175 | WT | Tg | 15 | 8 | 53.33% |
| 19 | VRF15164 #1 | x | VRF16916 B #275 | WT | Tg | 18 | 8 | 44.44% |
| 20 | VRF15164 #2 | x | VRF16918 c # 286 | WT | Tg | 21 | 10 | 47.62% |
| 21 | VRF18287 A #292 | x | CRF19714 #1 | Tg | WT | 17 | 9 | 52.94% |
| 22 | VRF18287 A #292 | x | CRF19714 #2 | Tg | WT | 16 | 9 | 56.25% |
| 23 | VRF18287 A #292 | x | CRF19714 #3 | Tg | WT | 16 | 8 | 50.00% |
| 24 | VRF18287 A #292 | x | CRF21575 C57 | Tg | WT | 10 | 6 | 60.00% |
| 25 | VRF22157 A #357 | x | CRF21575 C57 | Tg | WT | 13 | 7 | 53.85% |
| 26 | VRF22157 A #358 | x | CRF22650 C57 | Tg | WT | 6 | 3 | 50.00% |
| 27 | VRF22157 A #361 | x | CRF22650 C57 | Tg | WT | 12 | 7 | 58.33% |

**Table 3.5 Stability of IL-37aTg mice**

### 3.2.4.4 The organ expression level of IL-37a in IL37aTg mice

I next determined the tissue expression of IL-37a transgene in mice. Several important immune organs were collected from WT and IL-37aTg mice (3 each), including heart, liver, lung, gut, kidney, skin, brain and spleen. Total RNA was extracted and the expression levels of the IL37a in each organ were determined by qPCR and compared. As showed in Figure 3.19, no IL-37 expression was detected in the WT mice. All the organs from IL37atg mice had significantly high IL-37 expression compared to each of the WT control organs. Lung was the organ with highest IL-37 expression, followed by heart, gut, skin, liver, kidney, spleen and brain.

To further determine if the expression and production levels of IL-37a are inducible in the IL-37aTg mice, I collected serum and spleen samples from the 4 generations of IL37aTg mice (G0-G3) with or without LPS injection and compared their IL-37a mRNA and protein levels by qPCR and ELISA, respectively. As showed in Figure 3.20a, the expression of IL-37a in the transgenic mice was inducible by LPS injection. Furthermore, the expression levels of IL-37a in the 4 generation of IL37atg mice were comparable. The result suggests that the IL37a transgene is stable, at least in spleen. To further confirm the IL-37a transgene stability and the protein level of IL-37a in the transgenic mice, the IL-37a protein productions in the serum of the 4 generations of IL-37aTg mice were measured by ELISA kit. In consistent with the RNA levels, the IL-37a protein productions were also induced by LPS in the transgenic mice (Figure 3.20b). Different from the mRNA level, the serum IL-37a protein levels were slightly increased from GO to G4 of the mice.

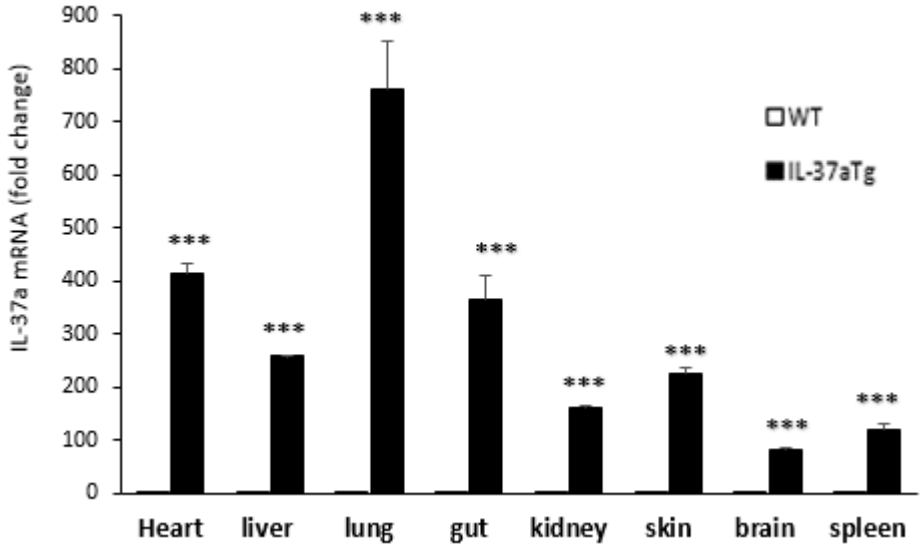



**Figure 3.19 IL-37 tissue distribution in IL-37aTg mice**

The organs from WT and IL-37aTg mice were collected and RNA extracted. The levels of IL-37a expression in each organ were measured by qPCR. The gene expression form all tissues of IL-37aTg are compared with WT as a control by student T-test. Data are means ± SD, and are representative of at least 3 mice/group. ***P<0.001 compared to control.

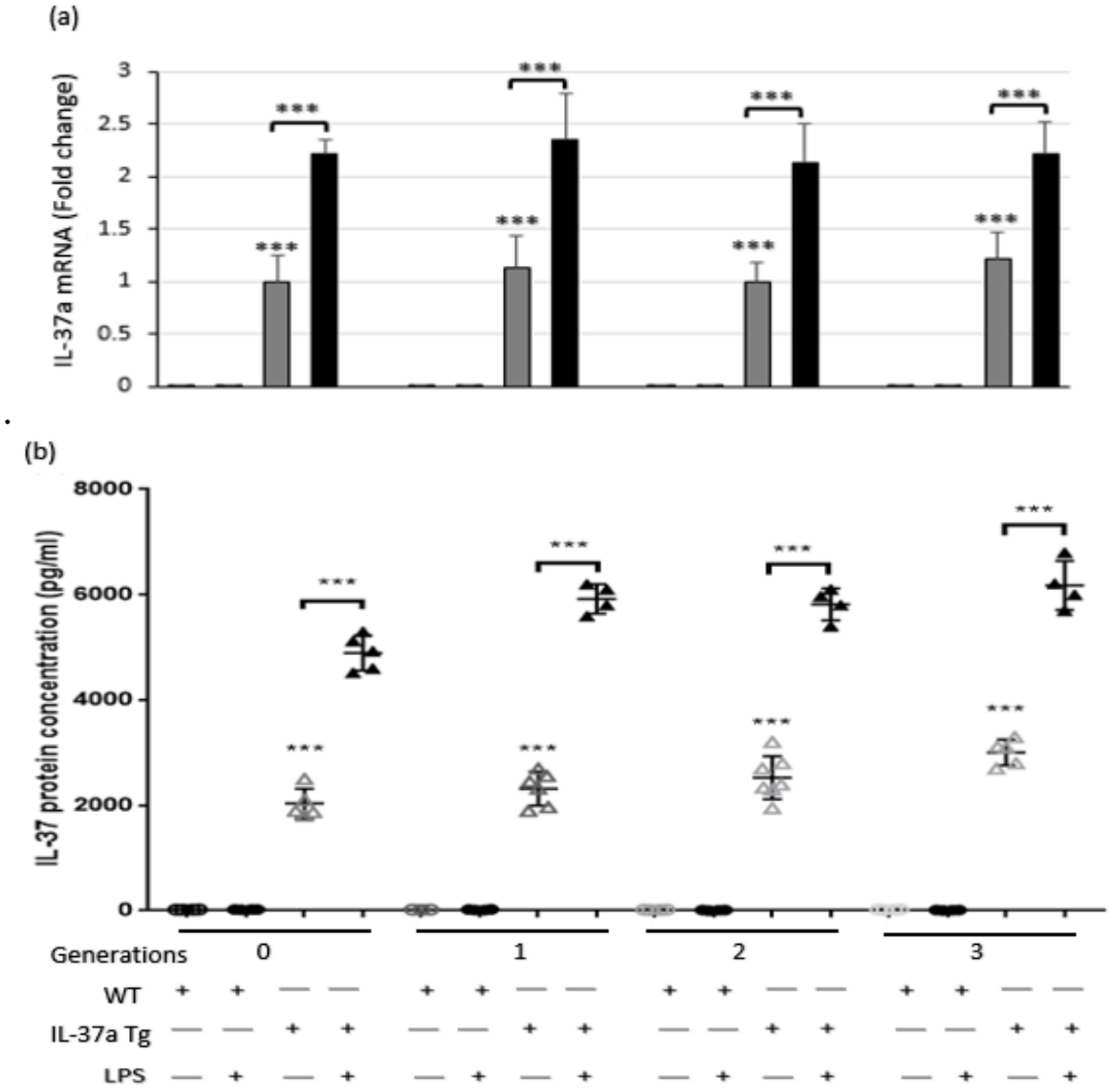



**Figure 3.20 Comparing RNA and protein levels of IL-37a in the 4 generations of IL37aTg mice**

Four generations (G0-G3) of IL-37aTg mice (around 4 mice/group) were injected or not injected with LPS (150ug/mice). a). The spleen samples were collected from the mice and the IL-37a mRNA level was measured by qPCR. Using T-test, data are means ± SD and are representative of at least 4 mice. ***P<0.001 compared to control. b). Serum samples were collected from the mice before and after LPS stimulation. The IL-37 protein level in serum samples was measured by IL-37 ELISA kit. Using T test, data are means ± SD and are representative of at least 4 mice. ***P<0.001 compared to control.

#### 3.2.4.5 Generation and identification of IL-37a receptor (IL-1R8) knockout mice

The IL-1R8-deficient (IL-1R8KO) mice were generated by homologous recombination (Garlanda *et al.*, 2004). An internal ribosome entry site (IRES)–LacZ cassette followed by the PGK-neomycin resistance gene was inserted into the downstream of the first ATG of IL-1R8 gene to abolish the gene expression (Figure 3.21a). The homo and heterozygous IL-1R8KO mice can be identified by genotyping using PCR (Figure 3.21b). The IL-1R8KO homozygous mice should display a 402bp band by PCR, the WT mice should have a 126bp band and the IL-1R8KO heterozygous should have both bands (Figure 3.21b). The IL-1R8KO mice can also be further identified in RNA levels by RT-qPCR (Figure 3.21c). The WT and IL-37aTg, but not the IL-1R8KO mice expressed IL-1R8 in splenocytes (Figure 3.21). We amplified the strain of mice by interbreeding with the homozygous male and female IL-1R8KO mice to produce homozygous mice for experiments.

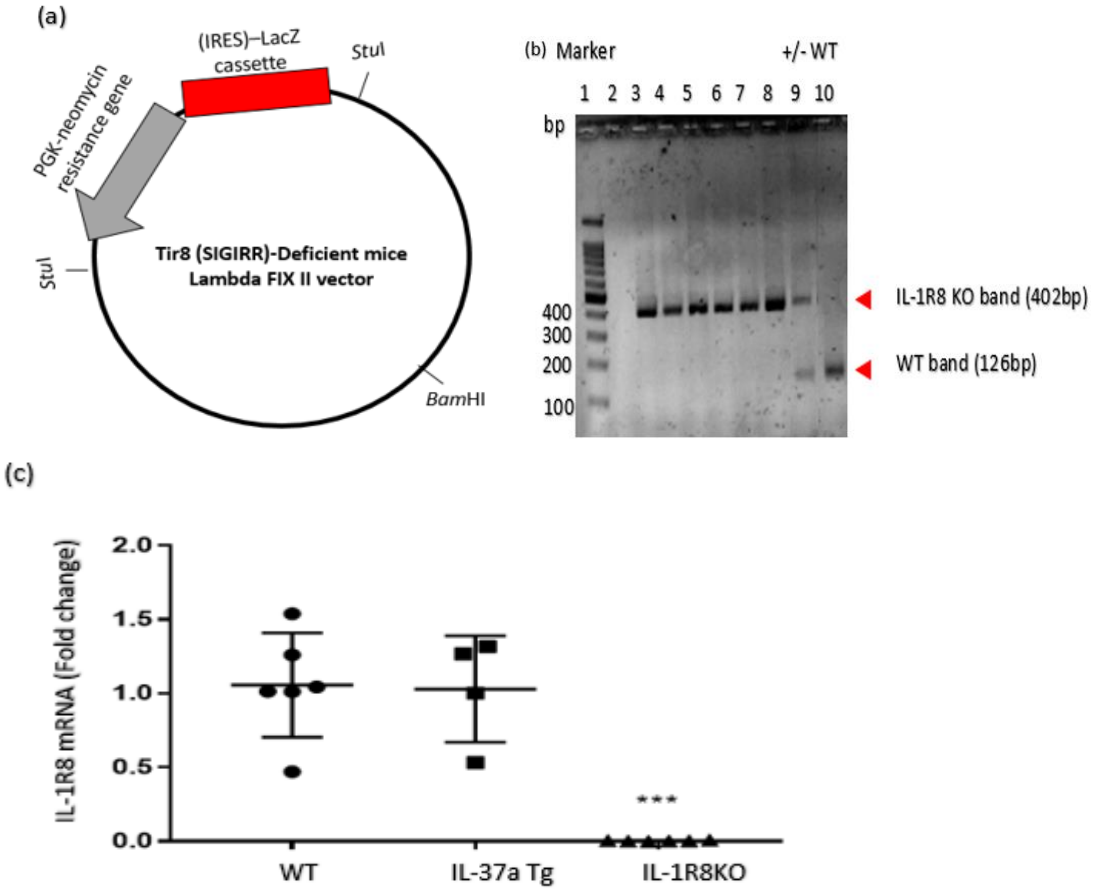



**Figure 3.21 The generation and genotyping of IL-1R8 deficient mice**

a) Schematic representation of IL-1R8 knockout mice construct. This construct built on Lambda FIX II vector, with an (IRES)-LacZ cassette to replace the IL-1R8 site. PGK-neomycin resistant site is the antibiotic resistant gene coding sequence.

b) Genotype detection of IL-1R8 knockout mice by PCR. Predicted IL-1R8KO band at 402bp, WT band at 126dp. line 1: 100bp DNA marker; Line 3-10: IL-1R8KO homozygous (-/-); Line 2: negative control; Line 9: IL-1R8KO heterozygous mice (+/-); Line 10: WT mice.

c) The splenocytes from wild type, IL-1R8KO and IL-37aTg mice were collected. The IL-1R8 mRNA level was measured by RT-qPCR. Using student T-test for analysis, data are means ± SD and are representative of at least 5 experiments. ***P<0.001 compared to control.

## 3.3 Discussion

In summary, we found for the first time that the dimerization of human IL-37a protein and the key amino acid are involved in the IL-37a protein. I also established the method for the successful production and purification of natural and mutant human IL-37a and b recombinant protein in *E. coli*. Finally, we generated IL-37aTg mice and obtained IL-1R8 deficient mice in order to study IL-37a function and receptor *in vivo*. These are invaluable tools and widely used in our laboratory and beyond for the study of IL-37a biology and function.

We demonstrated the dimerization of human IL-37a by native PAGE, molecular modelling and gene mutagenesis. Using the native PAGE, we found that natural IL-37a proteins existed in both the monomer and dimer forms, with majority of the proteins was dimer. We further identified amino acid Y59 in IL-37a protein as the key amino acid in the IL-37a dimerization by molecular modelling. Finally, we confirm that mutagenesis of IL-37a Y59A effectively prevents the dimerization of IL-37a. Thus, like IL-37b, IL-37a can also form homodimers. It is important to know whether this affects IL-37a function.

Intriguingly, IL-37a and b may also form heterodimers via their Try within each isoform, as suggested by molecular modelling. However, since the two isoforms are different in N-terminal sequence and in molecular size it is still unclear how stable the heterodimer can be. This needs to be further confirmed empirically.

We designed the constructs for the expression of both natural and mutant IL-37a and b protein produced in *E. coli*. We also established the protocol for the purification of these IL-37 proteins from *E. coli*. With the results presented in this chapter, I have successfully produced milligram level of highly purified both natural and mutant IL-37a and b proteins.

Despite the successful expression of IL-37 proteins, I found that the production of IL-37a was more problematic than IL-37b. This was mainly because IL-37a was less soluble than IL-37b in several commonly used buffers and can precipitate from the

solution during the dialysis. Therefore, several dialysis conditions have been tested and optimised.

The chemical analysis of the two IL-37 isoforms suggests that the lesser solubility of IL-37a than IL-37b may relate to its higher isoelectric point, 9.2 for IL-37a comparing with 6.1 for IL-37b. Thus, in order to make IL-37a soluble in a dialysis buffer with PH 7.5, the salts concentration has to be relatively lower. For instance, IL-37a was insoluble in buffer 1 (PH7.5) with 500 mM of NaCl, and it became soluble when we reduced the concentration of NaCl to 250mM. This suggests again that IL-37a is different from IL-37b in protein features.

The human IL-37a transgenic mice showed no obvious difference compared with C57BL/6 wild-type mice in birth-rates, sex ratios and body weights. No obvious changes were identified in the anatomic and histological analysis of major organs: heart, liver, spleen, kidney, intestine and brain of the mice at 10 weeks of age (data not shown). Moreover, there is no differences in the IL37aTg mice at 6-10 weeks of age compare with WT mice which are normally used in our experiments. This suggests that transgenic production IL-37a is safe with no observed influence on mice homeostasis.

Differing from the reported IL-37b transgenic mice that express IL-37b from CMV promoter, our IL-37aTg mice express IL-37a from the CAG promoter. The reason for using CAG promoter was because CAG is more effective than CMV in initiation of gene expression in mammalian cells (Koyama *et al.,* 2000). This is mainly due to the CAG promoter containing both CMV immediate early enhancer and an enhancer element from the intron of the chicken beta actin gene, which is highly conserved among vertebrates (Tardieu *et al.,* 2014). Indeed, previous report shows the serum level of IL-37a in the IL-37aTg mice is much higher than the reported level of serum IL-37b in the IL37bTg mice (Nold *et al.,* 2010). The CAG promoter seemed to work well in all tested cell populations from the Tg mice, including T, B cells and macrophages (data not shown). This should be valuable for studying the function of IL-37a in each of the immune and tissue cells.

As with IL-37bTg mice, the transgene IL-37a expression in mice was inducible by TLR signal, including LPS, and its expression was initiated with a constitutive (non-inducible) CAG promoter. This may be due to the IL-37 mRNA containing an

instability element which can be stabilised by inflammatory signal, including TLR signals (Dinarello *et al.*, 2016).

Altogether, I have successfully produced a sufficient amount of highly purified natural and mutant IL-37a and b recombinant proteins. We have also generated and bred IL-37aTg and receptor deficient mice. While it was very time consuming, I have learnt a great deal from this part of the work. I believe these techniques are very useful, not only for the next part of my work involved in the functional study of IL-37a and b, but also for the ongoing research projects in our lab.

# Chapter 4

# IL-37 regulates TLR response

# *in vitro* and *in vivo*

# 4. IL-37 regulate TLR response *in vitro* and *in vivo*

## 4.1 Introduction

Although the biology and function of IL-37b have been studied, the biology and effect of other IL-37 isoforms, especially IL-37a, are still poorly studied. By analyzing IL-37a structure and key protein domains, we believe IL-37a should have bioactivity which is similar but different from IL-37b. Both IL-37a and b share C'-terminal IL-1-like domain that is important for receptor binding and signaling (Nold-Petry *et al.*, 2015). Thus, as IL-37b, IL-37a may also bind to the receptor complex consisting of IL-1R8 and IL-18Ra chains for signaling, and thereafter has similar bioactivity. However, the different N-terminals may make them different in biology and activity. The unique N-terminal of IL-37a contains a putative nuclear localization sequence (NLS) and elastase cleavage site. Research in this group has found that the NLS is functional and can translocate FLIL-37a from cytoplasm into cell nucleus (unpublished result), thus, IL-37a is largely a nuclear protein. Different from other IL-37 isoforms that carry a caspase I cleavage site, IL-37a contains a putative elastase cleavage site. Caspase I can cleave FLIL-37a into mature IL-37a (Nold-Petry *et al.*, 2015). However, the maturation process of FLIL-37b seems to be not required for IL-37b secretion and function since both FL and mature IL-37b can be released from cells and both forms of IL-37b are bioactive (Gu *et al.*, 2015). The maturation of IL-37a and the importance of maturation in IL-37a function and secretion are unknown. Furthermore, both IL-37b and a can form homodimer in higher concentration (Ellisdon *et al.*, 2017 and Chapter 4). The IL-37b monomer is better than dimer in bioactivity (Eisenmesser *et al.*, 2019). However, the bioactivity of dimer and monomer of IL-37a is also unknown.

Intriguingly, IL-37 is selectively expressed in human but not mice (Nold et al., 2010). Thus, study of human specific IL-37 function may help to understand not only its function in human, but also the difference between human and mice in immunity and disease.

While mice lost IL-37 in evolution, the receptors IL-1R8 and IL-18Ra remain and can respond to human IL-37 (Nold-Petry *et al.*, 2015). Therefore, mice are an optimal choice to study the function of human IL-37 *in vitro* and in disease *in vivo*. The function of IL-37b in diseases has been investigated in IL-37b transgenic mice (McNamee *et al.*, 2011; Liu *et al.*, 2018). The function of IL-37a in diseases has not been explored.

Salmonellosis is a food-born disease caused by *salmonella* infection. *salmonella* leads to many manifestations of disease: enteric fever (typhoid fever), enterocolitis and systemic illness (Trebichavský and Šplíchalová, 2010). TLRs play a critical role in *salmonella* infection (Mittrucker *et al.*, 2002). Several TLR agonists from salmonella, including lipopolysaccharide (LPS, for TLR4), lipoprotein (TLR2) and flagella (TLR5) have been identified (*ibid*). After *salmonella* infection, host innate immune cells, including DC and macrophages recognize the salmonella-derived TLR ligands via their TLRs and trigger a protective Th1 immune response, producing IL-12 and IFN-γ to kill intracellular salmonellas (Trebichavský and Šplíchalová, 2010). several anti-bacteria substances are generated by this process, for instance nitric oxide, in order to assist in the removal of intracellular salmonella (Moss *et al.*, 2004). Thus, factors that regulate NO production are likely important in salmonella infection. TLR2,4 and 5 play a critical role in the early immune response by identifying the invaded pathogens, then activate host innate immune response (Roll *et al.*, 2007). While an appropriate level of TLR response is beneficial, dysregulation of TLR function may lead to inflammatory response, pathogenesis and death (Mittrucker *et al.*, 2002). Thus, TLR response in salmonella infection must be carefully controlled.

It has been noted that Salmonella infection is different in human and mouse, also in nitric oxide induction (Vazquez – Torres *et al.*, 2008). Salmonella typhi can only infect human but not mice (Crump *et al.*, 2004). The reason behind this is still less understood.

In Chapter 3, I have produced the monomer and dimer rIL-37a and b proteins. I also generated the IL-37aTg mice and introduced IL-IR8KO mice, with all these reagents

and mice strains, I studied here the function of IL-37a *in vitro* and in salmonella infection *in vivo*.

The main aims of this chapter were:

To investigate the induction and regulation of IL-37 isoforms under different TLR stimulations in human macrophages.

Determine the function of endogenous and exogenous IL-37a in the regulation of TLR response *in vitro*.

Investigate the function of IL-37a in Salmonella infection *in vitro* and *in vivo*.

We demonstrated here that IL-37a is highly induced among the IL-37 isoforms in TLR-stimulated macrophages. Both endogenous and exogenous IL-37a are bioactive and immunosuppressive in TLR-induced inflammatory response. The monomer IL-37a is more immunosuppressive than the dimer. Moreover, the IL-37a-mediated immune regulatory effect is IL-1R8 dependent.

While inhibiting inflammatory response, IL-37a also promotes salmonella infection in human and murine macrophages and in mice *in vivo*. This may be due to its regulatory effect on protective immunity and NO production. Thus, IL-37a is a previously unrecognized immunoregulatory cytokine which plays a regulatory role in TLR response and in infection.

## 4.2 Result

### 4.2.1 The induction of IL-37 isoform and receptor expression in macrophages by TLRs

The optimal induction and regulation of IL-37 isoform and receptor expression in macrophages is unknown. To investigate whether the IL-37 isoform and receptors are equally or differently regulated by TLR signals in macrophage, the human THP-1 monocytes were differentiated into macrophage first adding PMA, then stimulated by 4 different TLRs (LPS, PAM3, FLA-st and IMQ) for 12h and the IL-37 isoform and receptor expressions were determined by qPCR. As shown in Figure 1, in LPS and IMQ stimulated macrophages, the IL-37a, b and c were markedly induced, compared to IL-37d and e. However, in PAM-activated macrophages, both IL-37a and e were highly induced, IL-37c and d were less induced, while the IL-37b was not induced. In the case of FLA-st stimulated cells, all isoforms, except IL-37d, were significantly induced. These results suggested that IL-37 isoform expressions were selectively induced by different TLR signals and that among the IL-37 isoforms, IL-37a was highly induced and IL-37d was less induced in all the tested TLR stimulatory conditions.

the dynamics of IL-37 receptors (SIGIRR and IL-18Rα) expression in murine and human macrophages were also analysed by qPCR. Compared to the unstimulated control human macrophages, the IMQ, PAM3 and FLA-st, but not LPS significantly down-regulated IL-1R8 mRNA expression (Figure 4.2). PAM3, but not the other three TLR agonists enhanced the expression of IL-18Ra chain expression in human macrophages. In murine macrophage, PAM3 but not the other three tested TLR ligands also enhanced the expression of IL-1R8 (Figure 4.2), whereas all 4 TLR agonists, in particularly LPS, markedly elevated IL-18Ra expression. Thus, compared to murine macrophages, IL-1R8 expression in human macrophages was largely down-regulated by most tested TLR ligands.

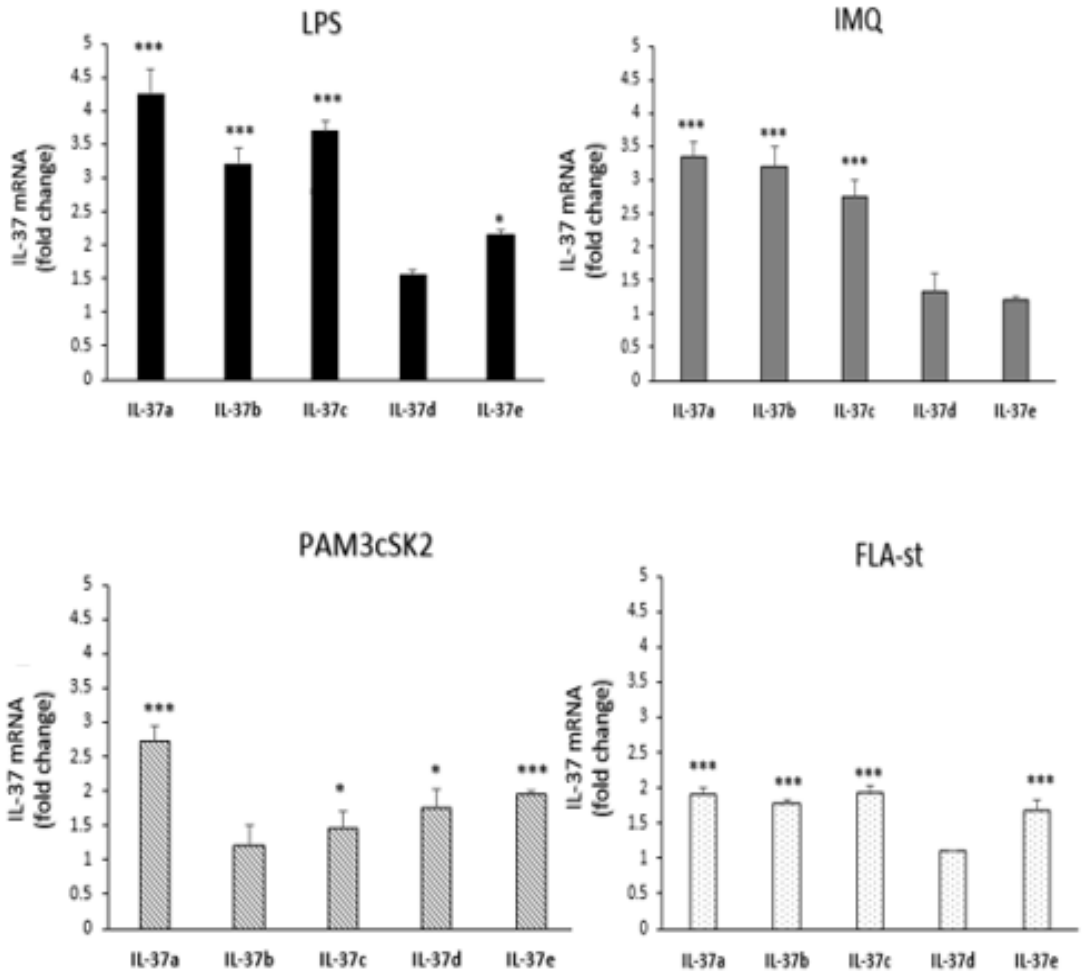



**Figure 4.1 The regulation of IL-37 protein isoform expression in human macrophages stimulated by TLR agonists**

Human macrophage THP-1 cells (1x10^6 cells/ml) were cultured and stimulated with LPS, IMQ, FLA-st and PAM3cSK2 (100ng/ml). The expression levels of IL-37 isoforms were measured by qPCR 12 hours after stimulation. All samples compared with negative control (Fold change = 1). Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to untreated cell controls.

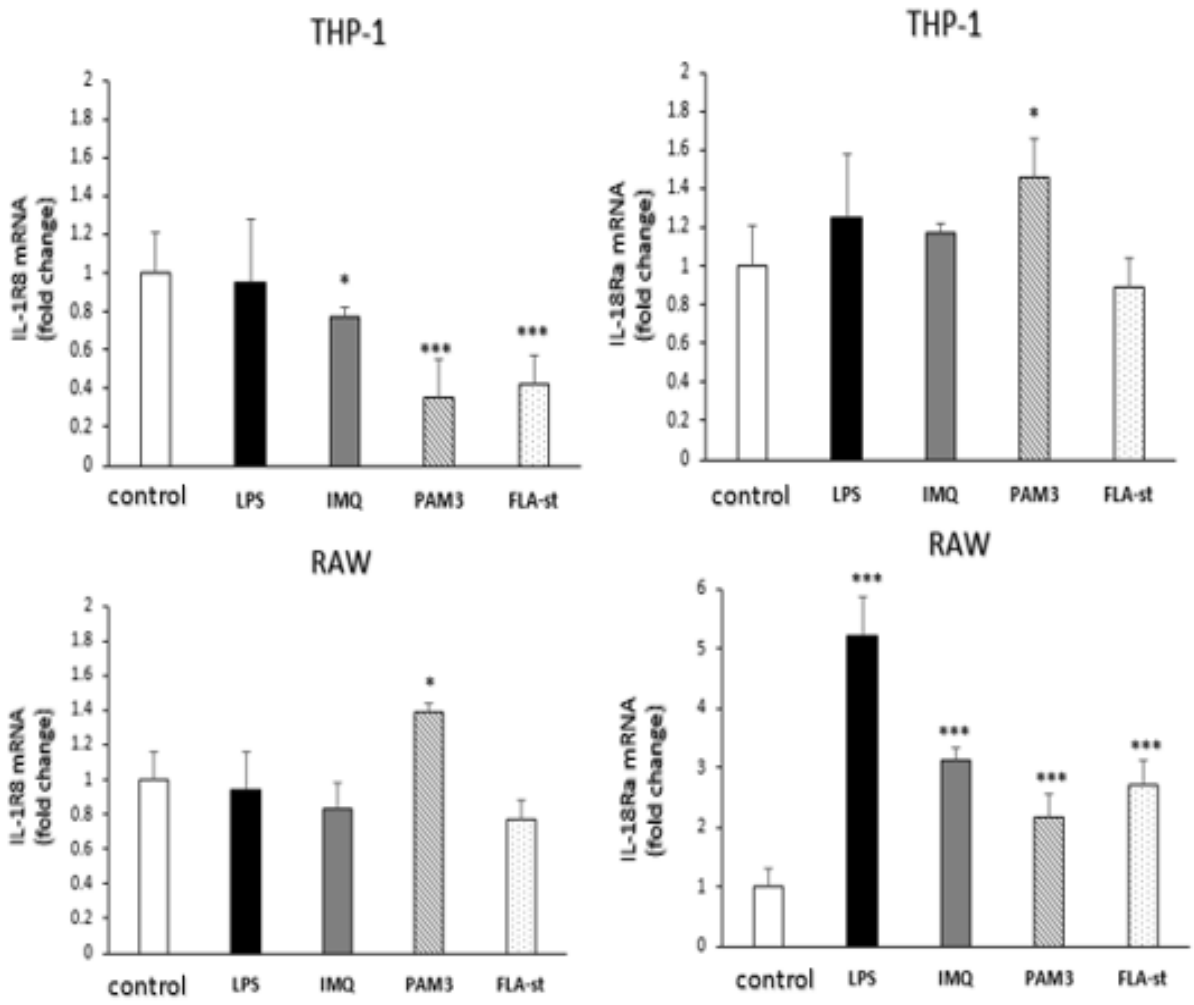



**Figure 4.2 The expression of IL-37 receptors in murine/ human macrophages stimulated by TLR ligands**

Human macrophage THP-1 cells and murine macrophage RAW cells (1x10^6 cells/ml) were stimulated with LPS, IMQ, FLA-st and PAM3cSK2 (100ng/ml) for 12 h. The levels of IL-37 receptors (IL1R8/IL-18Ra) were measured by qPCR. All samples compared with negative control (Fold change = 1). Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05 and ***P<0.001 compared to controls.

### 4.2.2. Knockdown IL-37a mRNA enhances inflammation cytokine production in human macrophages

Since IL-37a is highly inducible among the IL-37 isoforms by all tested TLR signals I sought next to study the function of IL-37a. To understand the function and importance of the endogenous IL-37a in the regulation of TLR response in human THP-1 macrophages, we knockdown the RNA of total IL-37 or IL-37a only in macrophages with specific siRNA. The siRNA for knockdown all IL-37 isoforms was designed to target the common exon 5 used by all IL-37 isoforms (siTotal IL-37) and siRNA for IL-37a (siIL-37a) targets its unique exon 3. The human THP-1 monocytes were differentiated into macrophage by adding PMA, then transduced with the control or specific siRNAs, respectively, for 48 hours. A scrambled siRNA was used as siRNA control. After that, the macrophages were stimulated with LPS for different times (6, 18 and 24 hours). The IL-37 isoform RNA and protein levels were measured by qPCR and ELISA. As shown in Figure 3, The knockdown of total IL-37 effectively dampened the expression of all IL-37 isoforms 18 hours after stimulation. IL-37a siRNA predominantly decreased the expression of IL-37a but, to a lesser extent, the IL-37e expression as well (Figure 4.3). Compared to the protein level of IL-37 in control siRNA group, siRNA knockdown either total IL-37 or IL-37a alone markedly reduced the production of IL-37 in culture supernatant, while the effect was more dramatic in the group of knockdown of total IL-37 (Figure 4.4). The result suggests that whereas the siRNA for total IL-37 isoforms was effective, the siRNA for IL-37a was less specific and also affected IL-37e. the reason is currently unclear. Since IL-37e only possesses part of the IL-1-like domain which is required for the receptor binding and signalling it is highly possible that IL-37e lacks suppressive activity (Boraschi *et al.*, 2011). We next compared the inhibitory effect between the total IL-37 and IL-37a knockdown groups on LPS-induced inflammatory cytokine expression in macrophages.

Compared with the control group, siIL-37a group significantly elevated the level of LPS-induced pro-inflammatory cytokines, IL-1α and IL-6 RNA expression and protein production (Figure 4.5a and b). The levels of inflammatory cytokines were further increased by the knockdown of total IL-37 (Figure 4.5a and b). At 36 hours culture,

no difference found between the two specific knockdown groups in the IL-6 protein enhancement (Figure 4.5b). Thus, these results suggest that endogenous IL-37a is bioactive and immunosuppressive. Our result also suggests that it is possible that IL-37-derived inhibitory effect on inflammatory cytokine production is largely mediated by IL-37a isoform.

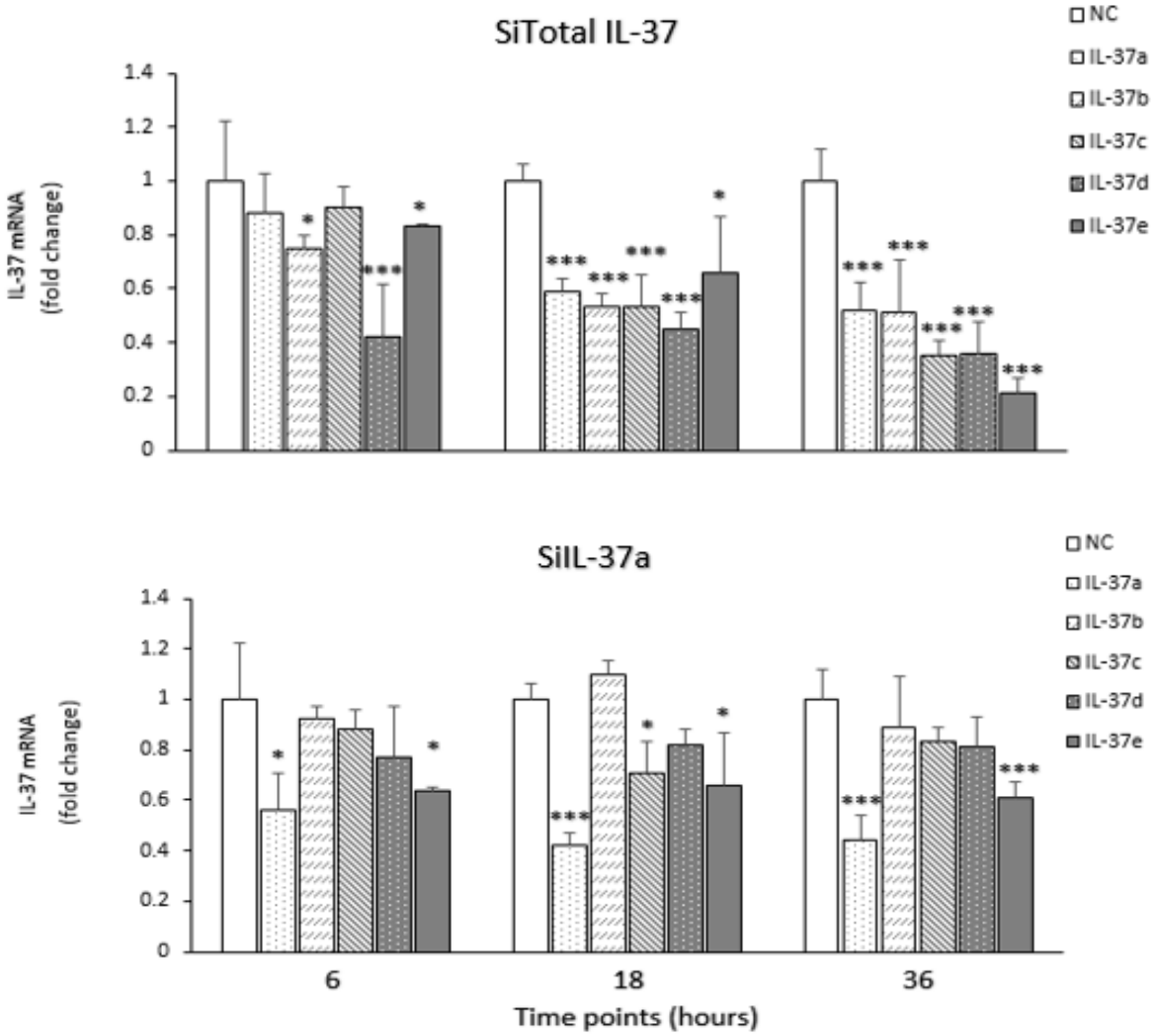



**Figure 4.3 The effect of Knockdown of total IL-37 and IL-37a by specific siRNA on the expression of IL-37 isoforms**

The total IL-37 and IL-37a RNA in human macrophages were knocked down by liposome-mediated siRNA transduction for 48 hours SiRNA sequence for IL-37, IL-37a and negative control (5pmol for each sample) mixed by Lipofectamine® RNAiMAX Reagent (1.5µl). After siRNA transduction, 1x10^6 cells were stimulated with LPS (100ng/ml) for 6, 18 and 36 hours. The RNA at each time point was isolated and the expression of five IL-37 isoforms were determined by qPCR. All samples compared with negative control (Fold change = 1). Using ANOVA test, data are means ± SD, and are representative of two independent experiments. *P<0.05 and ***P<0.001 compared to siRNA control.

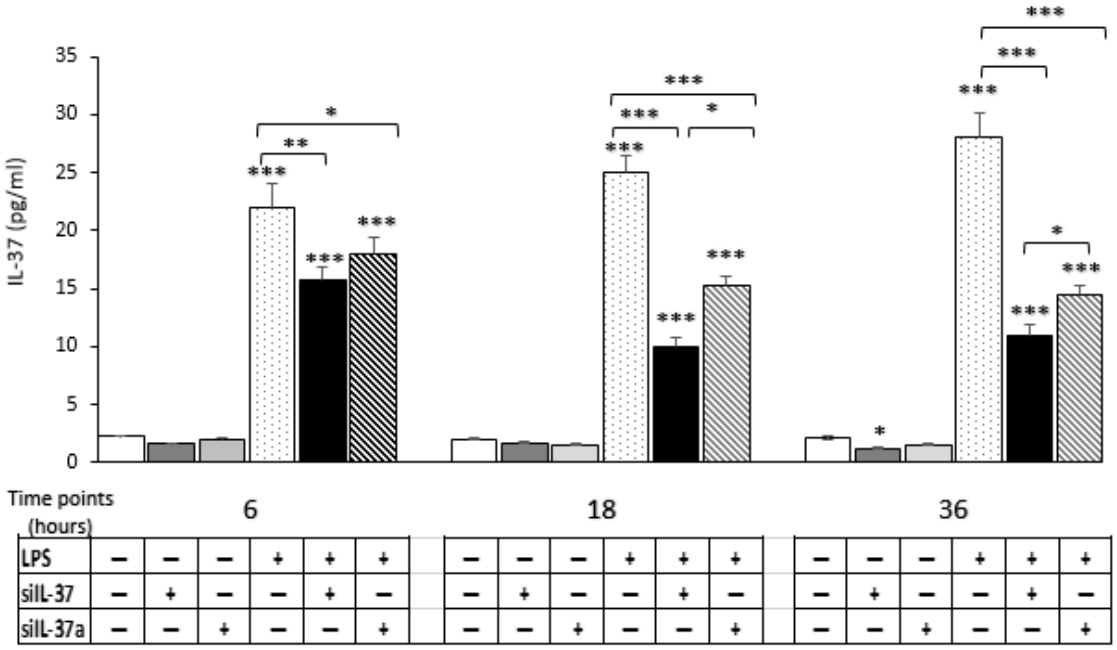



**Figure 4.4. siRNA for total IL-37 and IL-37a decrease the protein production of IL-37 in LPS-stimulated macrophages**

We also measured the production of IL-37 by ELISA. The total IL-37 or IL-37a RNA in macrophages were knocked down by specific siRNA transduction. 48 hours later, 1x10^6 macrophages were stimulated with LPS (100ng/ml) for 6, 18 and 36 hours. The production of IL-37 protein after siTotal IL-37 and siIL-37a treatments was determined by qPCR. In no LPS groups, all samples compared with negative control, in LPS groups, all samples compared with each other. Using ANOVA test, data are means ± SD, and are representative of two independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to siRNA control.

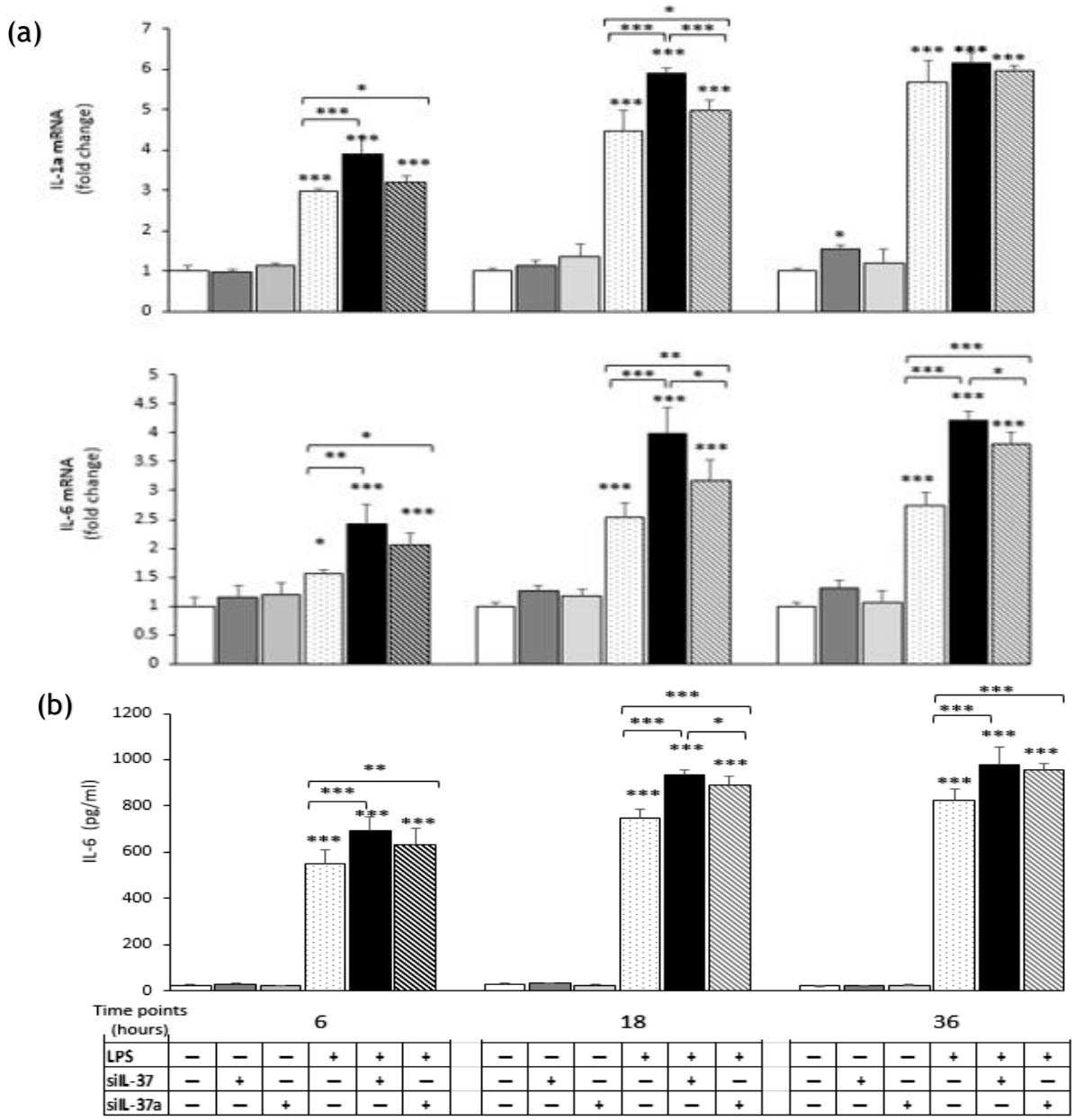



**Figure 4.5 Knockdown of IL-37a and total IL-37 by specific siRNA increases LPS induced pro-inflammatory cytokines production in macrophages**

Initially, total IL-37 and IL-37a RNA in THP1 cells were knocked down by specific siRNA as above. 48 hours after siRNA transduction, the cells were stimulated with 100ng LPS for 6,18 and 36 hours. a) Pro-inflammatory cytokine mRNA levels were determined by qPCR. b) Human IL-6 protein production was measured by ELISA. In no LPS groups, all samples compared with negative control, in LPS groups, all samples compared with each other. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

### 4.2.3. Recombinant IL-37a protein is immunosuppressive

I next investigated whether the exogenous IL-37 is bioactive and whether IL-37a and IL-37b are the same or different in bioactivity. We answered the questions by using recombinant IL-37a and b (rIL-37) proteins that I had produced before (Chapter 1). Firstly, I compared their capability in the inhibition of LPS-induced IL-6 production in murine macrophage RAW 264.7 cells (Figure 4.6). Macrophages were activated with or without LPS stimulation and treated with or without increased doses of IL-37a or IL-37b for 12 hours, the IL-6 production was measured by ELISA. As shown in Figure 4.6a, rIL-37a was immunosuppressive, which suppress LPS-induced IL-6 production in a dose-dependent manner. This is consistent with the function of endogenous IL-37a. Furthermore, rIL-37a was more effective than rIL-37b in the suppression of IL-6 production in macrophages. The best concentration for their inhibitory effect was 50 ng/ml. I further compared the dose and time-effect of rIL-37a and b in the context (Figure 4.6b). Macrophages were stimulated with LPS as above and treated with rIL-37a and b (10, 50 and 100 ng/ml) for 6, 12 and 24 hours as indicated. The bioactivity of IL-37a and b in suppressing LPS-mediated IL-6 production was measured and compared. The best time point for both IL-37 and b to suppress LPS-induced IL-6 secretion started from 12 hours. Again, the best dose for both cytokines to inhibit IL-6 production was the 50 ng/ml and IL-37a was more suppressive than IL-37b (Figure 4.6b).

According to the study of Ellisdon *et al*. (2017), the only form of IL-37 in human serum is monomer instead of dimer. Moreover, the natural IL-37 protein exists in a monomeric form and only the monomer but not the homodimer is bioactive and current recombinant IL-37 proteins are largely homodimer (Eisenmesser *et al.*, 2019). Therefore, I use mutant-monomer IL-37 in our experiment to mimic the condition of IL-37 in human serum and seek for better bio-activity.

### 4.2.4. IL-37a monomer is more effective than dimer in immunosuppression

Since it has been reported that the monomer form of IL-37b is more active than the dimers, I next investigated if this was also the case for IL-37a using the natural (dimer) and mutant (dimer) rIL-37a and b. I also compared the bioactivity of

monomer and dimer of rIL-37a and b in the same system of LPS-induced IL-6 production in macrophages as above. We found that monomer form of IL-37a was more suppressive compared to the dimer in LPS-induced IL-6 expression (Figure 4.7a) and protein production in macrophages in dose-dependent manner (Figure 4.7b), this was also the case for dimer and monomer IL-37b as reported (Figure 4.7a and b). In addition, similar to the bioactivity of natural IL-37a and b, the monomer form of IL-37a was also better than the monomer IL-37b in the inhibition of IL-6 production in this experimental context. Thus, monomer form of rIL-37a and b were utilised for the subsequent *in vitro* and *in vivo* experiments.

### 4.2.5. IL-37a also signals via IL-1R8

In order to define whether IL-37a also signals via the same receptor (IL-1R8) as IL-37b, I determined the bioactivity of IL-37a in splenocytes from IL-1R8 deficient (IL-1R8KO) mice. Briefly, the splenocytes from WT and IL-1R8KO mice were stimulated with or without LPS or IMQ and treated or not treated with IL-37a and b, respectively. Their effect on the inflammatory cytokines—IL-1α, IL-12 and IFN-γ expressions were quantified by qPCR. As shown in Figure 4.8, both IL-37a and b inhibited all three cytokine expressions in LPS or IMQ-stimulated WT splenocytes, compared with the controls. In contrast, both IL-37a and b lost their suppressive activity in the cells from IL-1R8KO mice. The result confirmed that the same as IL-37b, IL-37a also signals via IL-1R8.

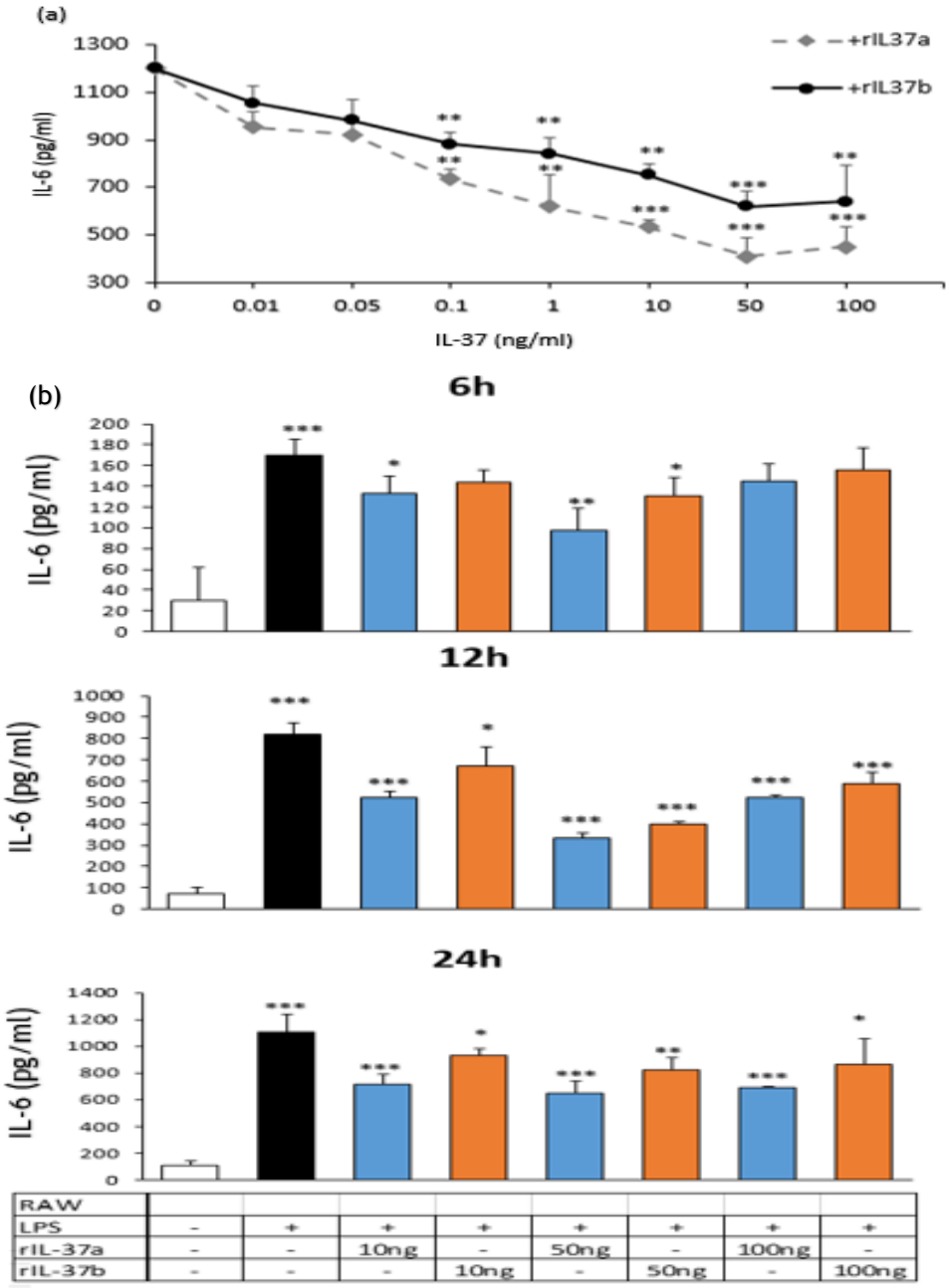
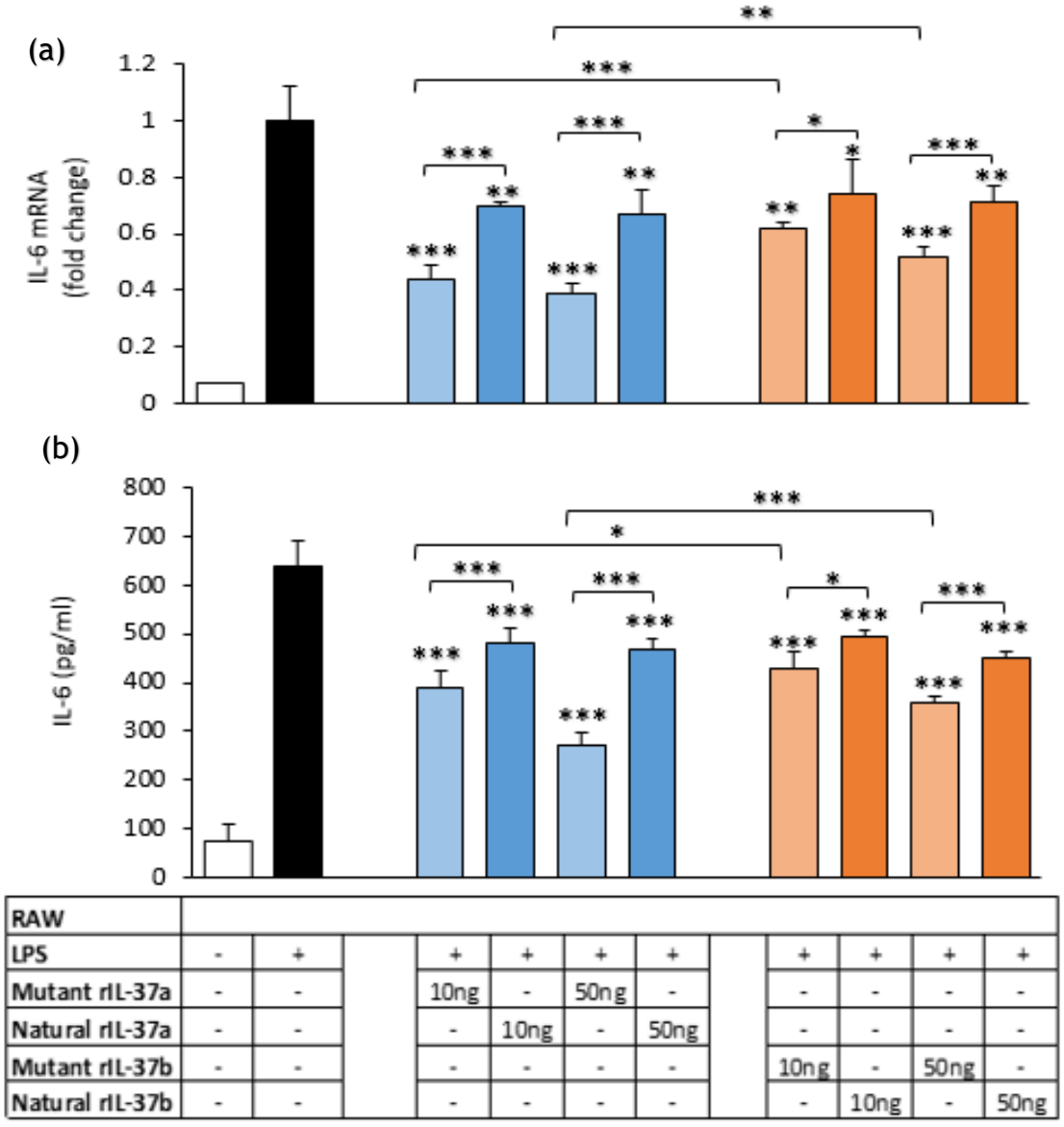



**Figure 4.6 rIL-37a is bioactive and more effective than IL-37b in the inhibition of LPS-induced IL-6 production *in vitro***

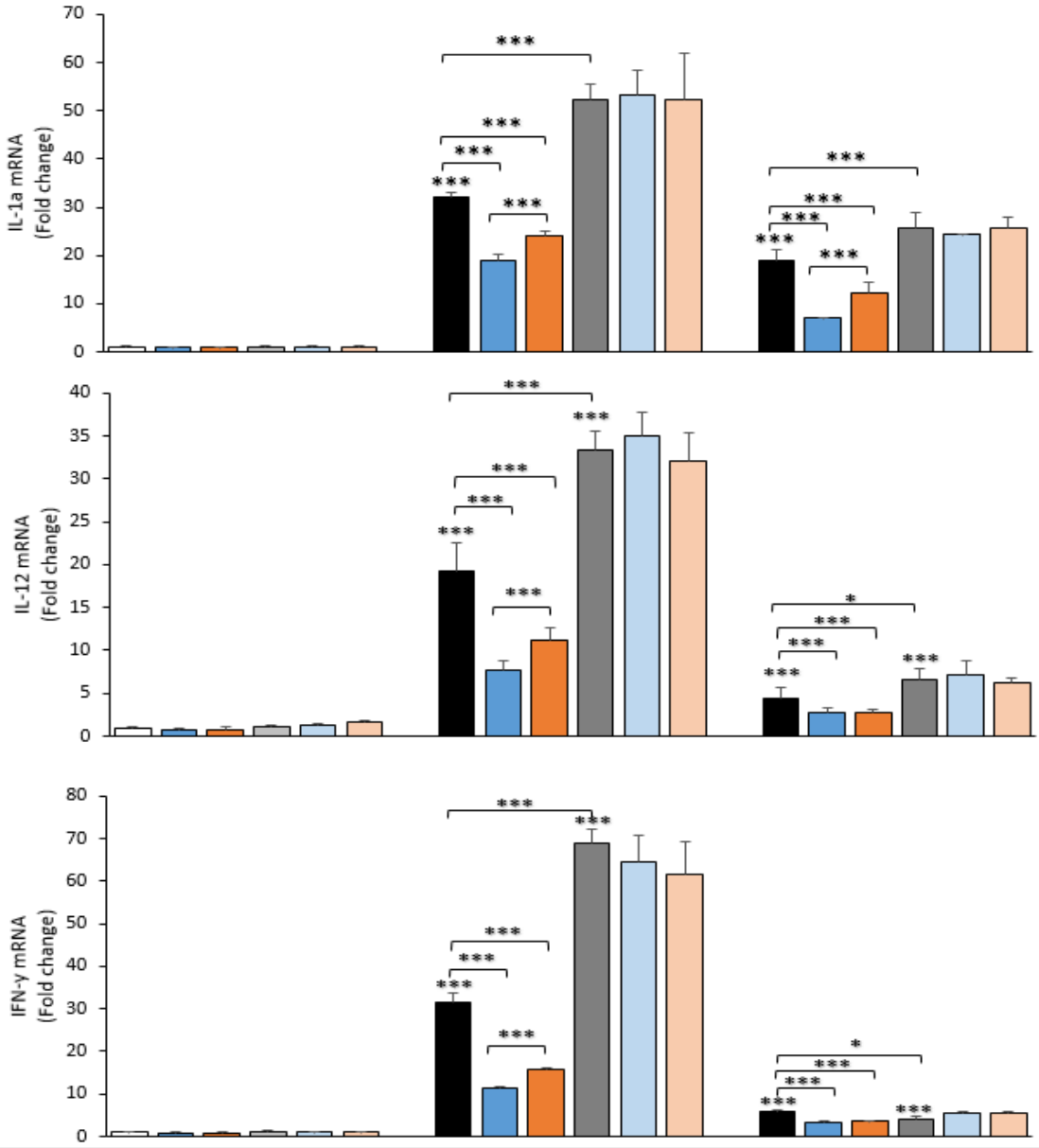
RAW cells (1x10^6/ml) were stimulated with 100ng/ml of LPS in the presence or absence of rIL-37a or b. The IL-6 levels in culture supernatant were measured by ELISA kit. a) The dose-dependent effect of IL-37a and b on the suppression of IL-6 production in LPS stimulated RAW cell. b) The time-effect of recombinant IL-37a and b proteins in IL-6 production. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. **P<0.01 and ***P<0.001 compared to controls.




| RAW | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS | - | + | + | + | + | + | + | + | + | + | |
| Mutant rIL-37a | - | - | 10ng | - | 50ng | - | - | - | - | - | |
| Natural rIL-37a | - | - | - | 10ng | - | 50ng | - | - | - | - | |
| Mutant rIL-37b | - | - | - | - | - | - | 10ng | - | 50ng | - | |
| Natural rIL-37b | - | - | - | - | - | - | - | 10ng | - | 50ng | |

**Figure 4.7 IL-37a and b dimers are more suppressive than the dimers in LPS-induced pro-inflammatory cytokine production**

Murine macrophages (RAW cells, 1x10^6/ml) were cultured in the presence or absence of 100ng/ml of LPS and treated with or without different doses of natural (dimer) or mutant (dimer) of IL-37a or IL-37b for 12 hours. a) The mRNA level of IL-6 in activated macrophages treated with mutant and natural IL-37a and b was determined by qPCR. b) IL-6 protein level in cell culture supernatants measured by ELISA. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.




| Splenocyte | control | | | | | | | LPS | | | | | | | IMQ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | + | + | + | — | — | — | | + | + | + | — | — | — | | + | + | + | — | — | — |
| IL-1R8KO | — | — | — | + | + | + | | — | — | — | + | + | + | | — | — | — | + | + | + |
| Mutant rIL-37a | — | + | — | — | + | — | | — | + | — | — | + | — | | — | + | — | — | + | — |
| Mutant rIL-37b | — | — | + | — | — | + | | — | — | + | — | — | + | | — | — | + | — | — | + |

**Figure 4.8 IL-37a signals via IL-1R8**

Splenocytes from WT and IL-1R8KO mice (1x10^6/ml) were cultured in the presence or absence of 100ng/ml of LPS or IMQ and natural or mutant IL-37a and b (50ng/ml) for 12 hours. Total RNAs were extracted from total cell lysate and the expression levels of pro-inflammatory cytokines (IL-1α, IL-6, IL-12 and IFN-γ) were measured by qPCR. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

### 4.2.6. IL-37aTg cells suppress TLR-induced inflammatory response *in vitro*

To further determine the regulatory role of human IL-37a in TLRs response in primary immune cells *in vitro* and in disease *in vivo*, we generated human IL-37a transgenic mice as described in Chapter 3. Initially, we tested the function of splenocytes from IL-37aTg mice in the regulation of TLR-induced IL-37a, receptor IL-1R8 and inflammatory mediator production *in vitro*. The splenocytes of IL-37aTg and WT mice were collected and cultured with or without LPS or IMQ stimulation for 12 and 24 hours. RNA was extracted from cultured cells for IL-37a, IL-1R8 and inflammatory cytokine detection by qPCR. As shown in Figure 4.9, we can only detect IL-37a in IL-37aTg mice not in WT, while the cells from both the WT and Tg mice expressed similar levels of IL-1R8 at 12 hours after culture with or without TLR stimulation. At 24 hours after IMQ stimulation, the IL-37a expression of Tg mice is reduced compared with 12 hours; there is no significant change of IL-37a expression in LPS stimulate group. Moreover, LPS but not IMQ cause the increase IL-1R8 gene expression in WT cells. However, this increase cannot be observed in Tg group. Furthermore, IMQ may leads to a decrease of IL-1R8 gene expression (Figure 4.9).

More importantly, the IL-37aTg cells dramatically reduced the expression of all tested inflammatory cytokines, IL-1α, IL-6, TNF-α, IL-12 and IFN-γ induced by either LPS or IMQ, compared to the WT control cells, in time-dependent fashion (Figure 4.10). Altogether, IL-37aTg mice are functional and immunosuppressive as the recombinant IL-37a.

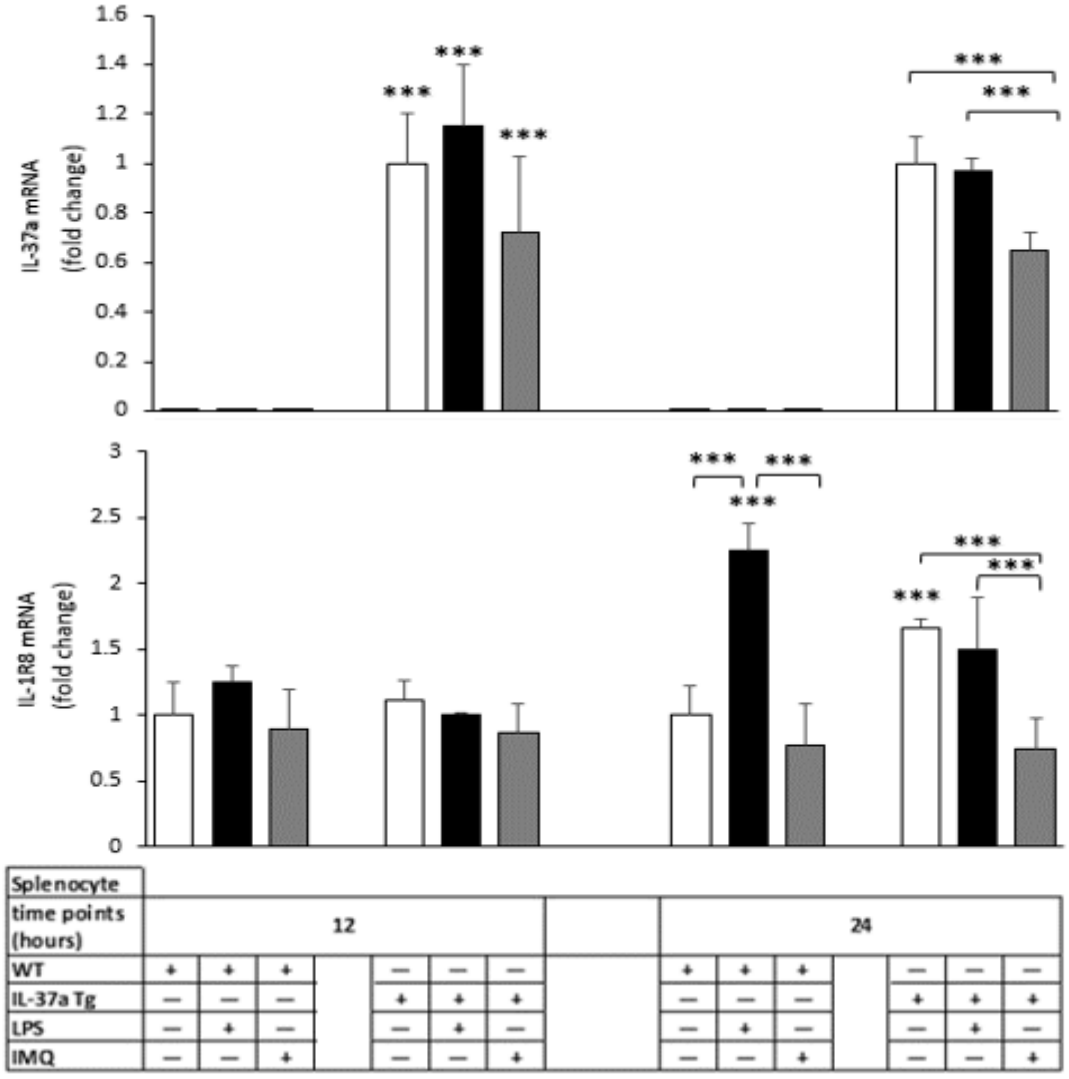
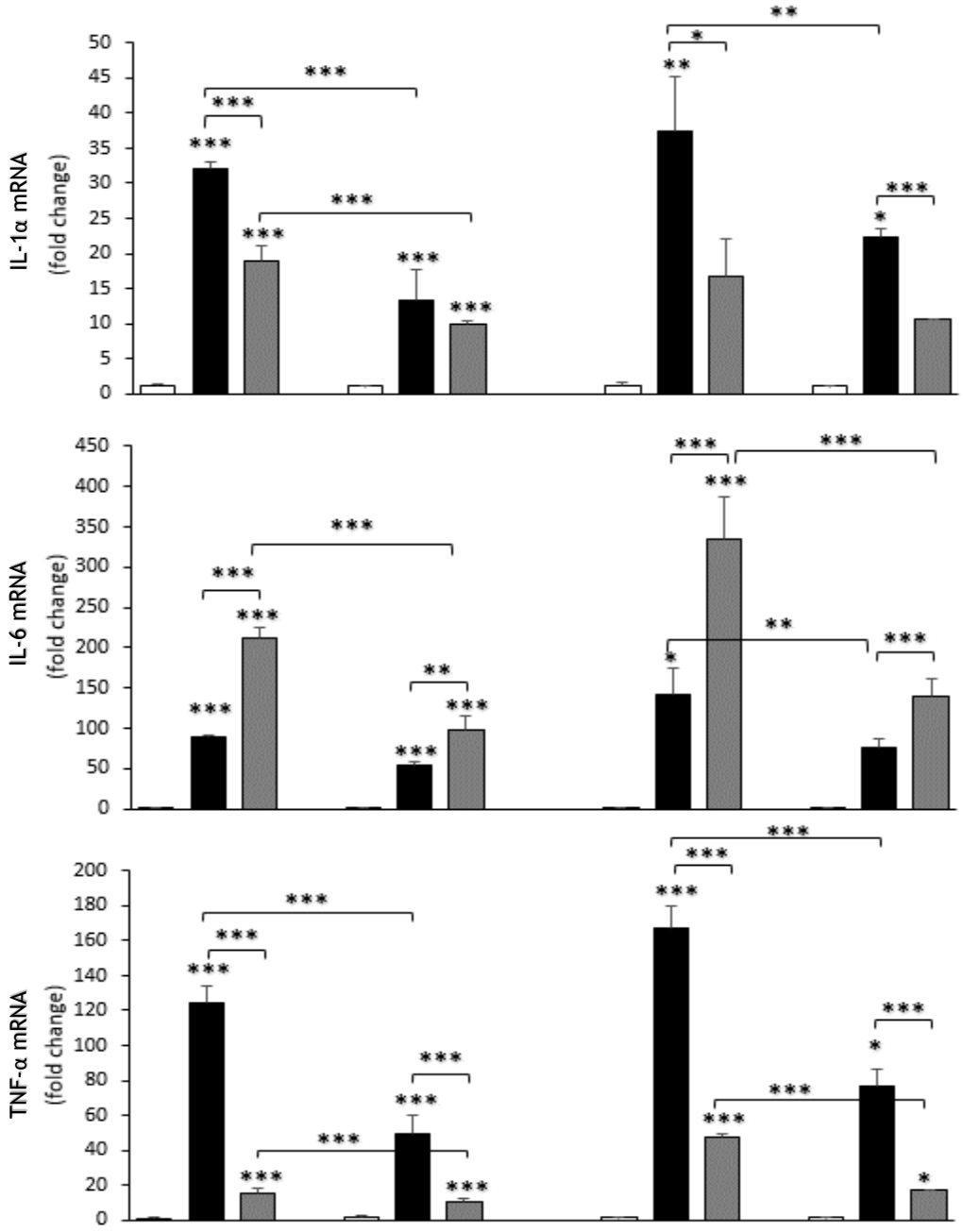



**Figure 4.9 Determining the expression levels of IL-37a and receptor in human IL-37a transgenic mice**

The splenocytes from IL-37aTg and WT mice (1x10^6/ml) were stimulated with 100 ng/ml of LPS or IMQ for 12 or 24 hours. The mRNA level of IL-37a and IL-1R8 in the cultured cells were quantified by qPCR. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.




| Splenocyte time points (hours) | 12 | | | | | | | | 24 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | + | + | + | | — | — | — | | + | + | + | | — | — | — |
| IL-37a Tg | — | — | — | | + | + | + | | — | — | — | | + | + | + |
| LPS | — | + | — | | — | + | — | | — | + | — | | — | + | — |
| IMQ | — | — | + | | — | — | + | | — | — | + | | — | — | + |

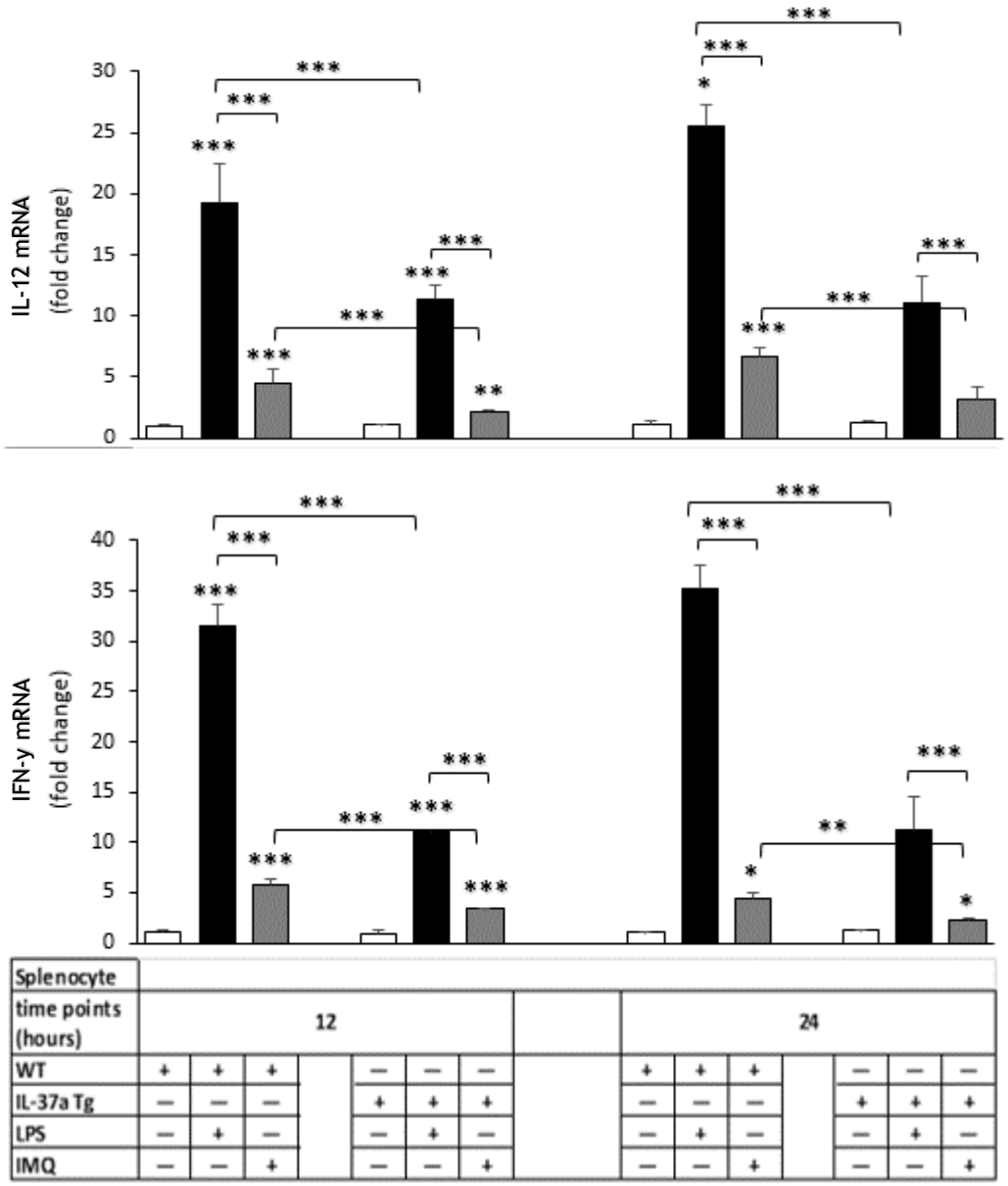



**Figure 4.10 IL-37aTg cells suppress the expression of pro-inflammatory cytokines induced by TLRs**

The splenocytes from IL-37aTg and WT mice (1x10^6/ml) were stimulated with 100 ng/ml of LPS or IMQ for 12 or 24 hours. The mRNA level of pro-inflammatory cytokines IL-1α, IL-6, TNF-α, IL-12 and IFN-γ in the cultured cells were quantified by qPCR. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

### 4.2.7. IL-37a is highly induced by *salmonella* infection in human macrophage

Since we found that IL-37a has the capability to suppress TLR ligand-induced inflammatory response, including the salmonella-derived TLR ligands, TLR4 ligand LPS, TLR2 ligand lipoprotein and TLR5 ligand flagellin, as a part of our research project, I sought next to study its role in *salmonella* infection *in vitro* and *in vivo*. In order to understand the involvement of IL-37 in salmonella infection, I primarily determined the induction of IL-37 isoform in salmonella infected macrophages.

Human THP-1 monocytes were differentiated into macrophages by PMA and culture in complete RPMI medium overnight before infection. The cells were infected with *Salmonella Typhimurium* bacteria (strain 1344) for different times and IL-37 isoform expression analysed by real-time PCR. As showed in Figure 4.11, all 5 of IL-37 isoforms were induced from 6 h after salmonella infection in macrophages. Among the isoforms, IL-37a was the most induced isoform, followed by IL-37c, b, e and d at 12 h. 24 h after infection, IL-37c was the dominant and IL-37d still was the lowest. In general, IL-37 expression is induced by salmonella infection in human macrophages and IL-37a was one of the highly induced IL-37 isoform, therefore, our subsequent experiments focused on IL-37a in salmonella infection.

### 4.2.8. Salmonella infection is different between murine and human macrophages

The next question we asked was: what is the difference between human that express IL-37 and murine macrophages which do not, in salmonella infection?

Both human and murine macrophage cell line were cultured in the same way, in the same medium. Same number of murine and human macrophages (1x10^6/ml) were infected with 10^8 of *salmonella* in the same time for up to 24 h. After infection, the bacterial numbers in the macrophages were counted under microscope and displayed as clone formation unit (CFU) count, as shown in Figure 4.12a and b. One hour after salmonella infection, murine macrophages contained more bacteria than human cells. However, the bacterial number was markedly reduced in murine cells 6 h after infection, while the bacterial number in human cells was increased. At 12h

and 24 h, no bacteria were found in infected murine macrophages, in contrast, the bacteria were still observed in human cells (Figure 4.12a and b). The result suggests that while salmonella infects murine macrophages more easily than human cells, they survive longer in human macrophage than in murine macrophage.

Nitric oxide (NO) is one of the main bactericidal factors in intracellular *salmonella* infection. NO is generated from arginine in the presence of the inducible NO synthase (iNOs). iNOs is largely induced by inflammatory signals, including TLR, inflammatory cytokines, IFN-γ, IL-1 and IL-6 in macrophages (Jin *et al.*, 2016). I investigated next whether the difference in salmonella infection between human and murine macrophages was due to their difference in NO production. In the same experiment, the levels of iNOs expression and related NO production in the infected murine and human macrophages were measured and compared. in the comparison with uninfected cell control group, salmonella infection enhanced iNOs expression in both murine and human macrophage and with same kinetics; peaked at 12 hours and declined after that (Figure 4.13a). However, the quantity of NOs induction varied between human and murine macrophages. The iNOs expression was 3-4 folds higher in murine macrophages than in human cells (Figure 4.13a). this was further confirmed by the NO measurement in the culture supernatants (Figure 4.13b); Infected murine macrophages that expressed higher iNOs, also produced more NO than human cells. Intragluteally, the NO levels were negatively correlated with the bacteria count in Figure 4.12.

### 4.2.9. Salmonella infection induces less inflammatory-related cytokines in human than murine macrophages

As iNOs expression can be upregulated by proinflammatory cytokines, IL-1, IL-6 and downregulated by anti-inflammatory cytokines, including IL-10, we determined and compared the key cytokine expression profiles between human and murine macrophages in the same infection experimental context. The transcription of 7 pro-inflammatory cytokines and one anti-inflammatory cytokine, IL-10, were determined by qPCR. As shown in Figure 4.14, in infected murine macrophages, all the tested cytokines were induced from 6 h after infection, but with different kinetics. The most induced cytokines were IL-6 followed by IL-1α and IL-1b. However, in human THP-1 cells, the most induced cytokine was the anti-inflammatory cytokine IL-10. The inflammation-related cytokines IL-6, IL-12 and IFN-γ were only marginally induced and IL-1α, IL-1ß, IL-33 and TNF-a were largely uninducible in the infected human macrophages, compared to those in murine cells (Figure 4.14). In summary, salmonella infection in human macrophages lead to dominant IL-10 expression and reduced inflammatory cytokines and NO production, thereby prolonging infection, compared to that in murine cells.

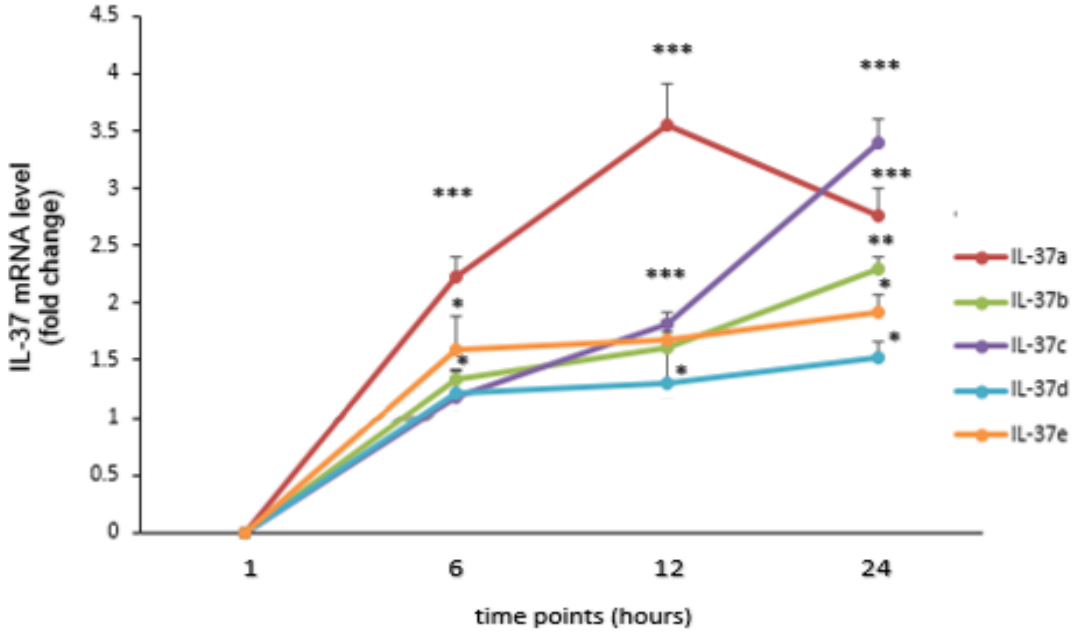



**Figure 4.11 IL-37a is the dominate IL-37 isoforms induced by *salmonella* infection in macrophages**

Human THP-1 monocytes were differentiated into macrophages by PMA and cultured in complete RPMI medium overnight before infection. Macrophages(1x10^6/ml) were infected with *salmonella* (SL1344 strain, 1x10^8/ml). The kinetic expression of IL-37 isoforms in macrophages was determined by qPCR 1,6,12 and 24 hours after infection. All samples compared with negative control (fold change = 1). Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

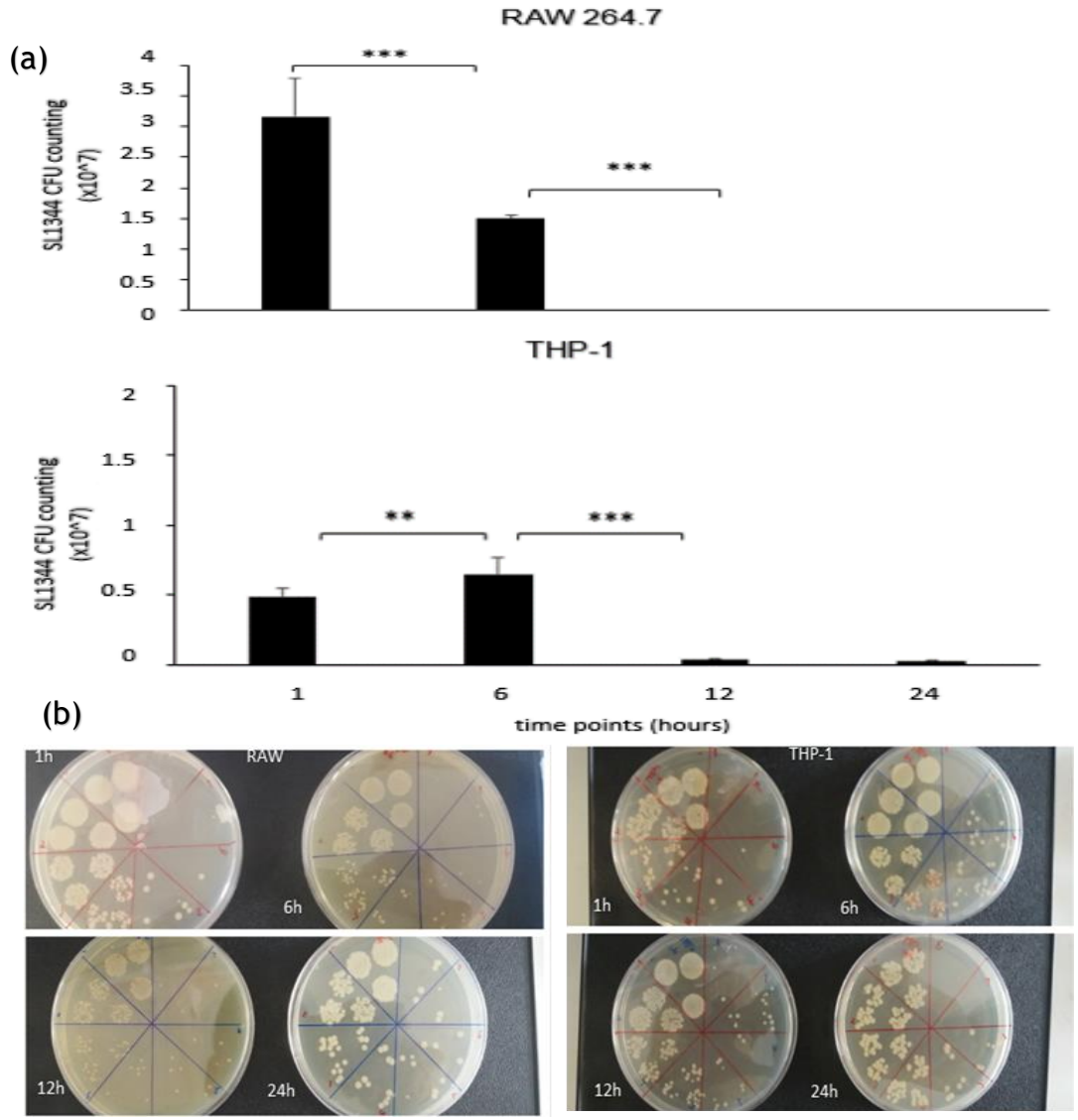



**Figure 4.12 *Salmonella Typhimurium* survive longer in human macrophages compared to murine cells**

THP-1 macrophages and RAW cells (1x10^6/ml) were infected with SL1344 bacteria(1x10^8/ml). The cells were collected 1, 6, 12 and 24 hours after infection. The cells were harvested in lysis buffer and sonicated to release the intracellular bacteria. The cell lysis was diluted eight times and incubated on LB agar plate 37℃ for 24 hours. The bacterial numbers in each of the samples were counted using spot plate and displayed as Colony-forming unit (CFU). a) The bacterial counts in infected THP-1 and RAW cells at different time points. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls. b) Pictures of serial dilutions of bacterial colony in spot plate of *salmonella* infected human and murine macrophages (24h picture result is different from the CFU).

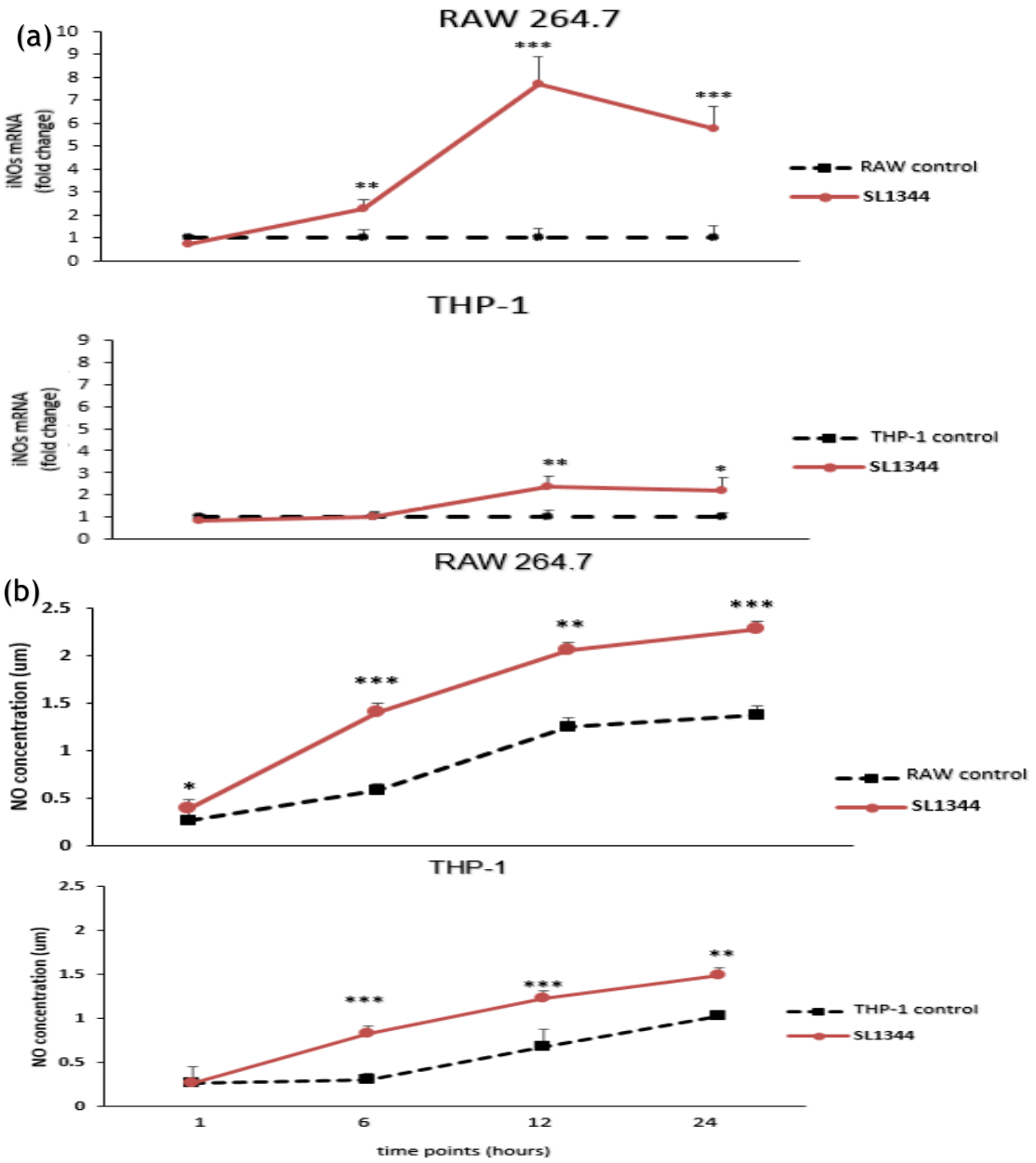



**Figure 4.13 Human macrophages reduce iNOs expression and NO production compare with murine cell after Salmonella infection**

Human THP-1 macrophage and murine RAW macrophage cells were cultured in 1x10^6 cell/ml and infected with SL1344 infection (1x10^8/ml) for 1,6,12 and 24 hours. Cells and culture supernatants were collected from each time point. The iNOs mRNA level were measured by qPCR and NO production in cell culture supernatant was measured by Griss Reagent kit. a) Comparison of iNOs expression in THP-1 and RAW cells. b) Comparison of NO production in THP-1 and RAW cells. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

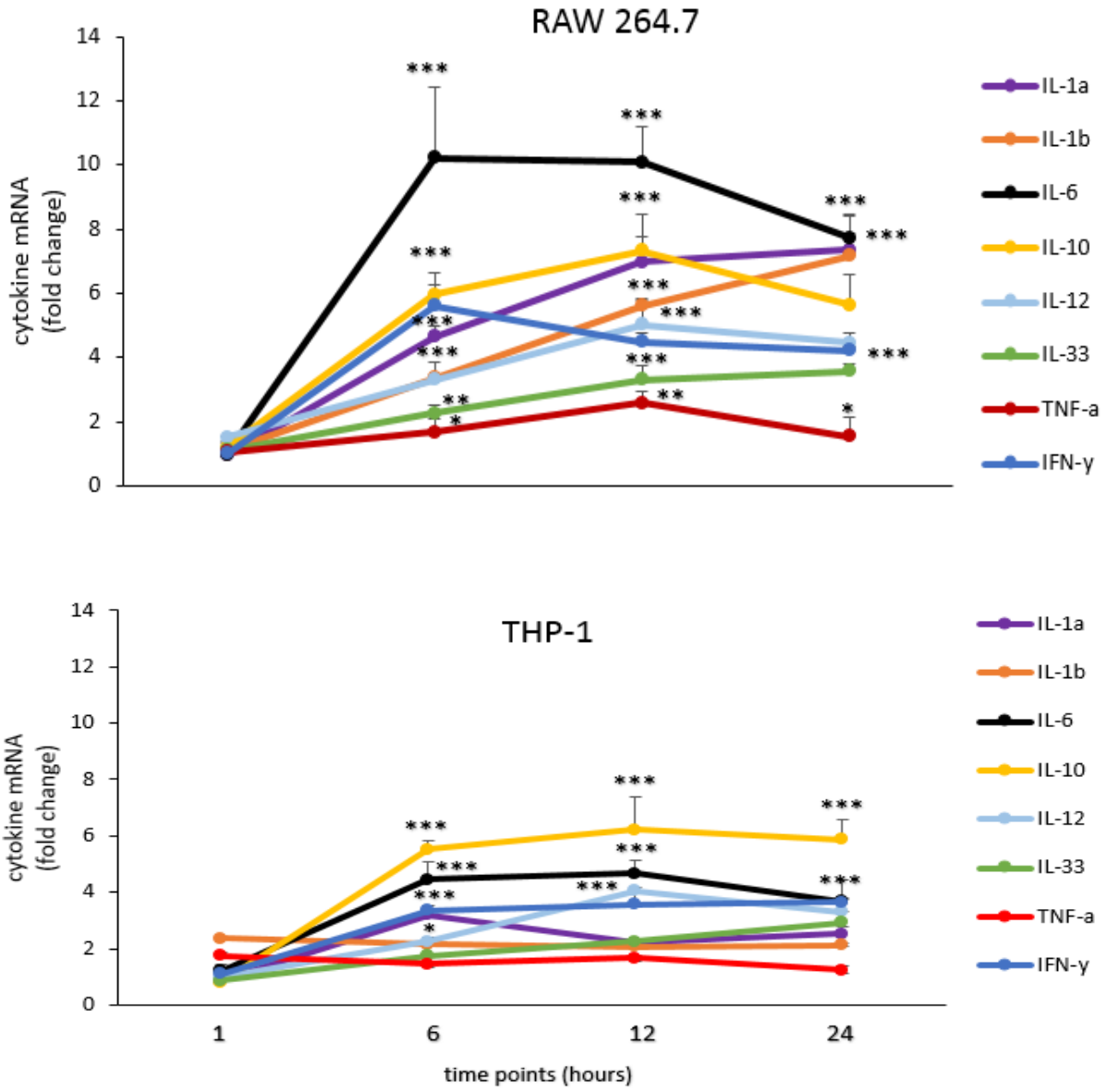



**Figure 4.14 Differential induction and expression of anti- and pro-inflammatory cytokines in salmonella infected human and murine macrophages**

Human THP-1 and murine RAW macrophage cells were cultured in 1x10^6 cell/ml and infected with Salmonella (1x10^8/ml) for 1,6,12 and 24 hours. Cells were collected and RNA isolated. The mRNA levels of inflammation-related cytokines (IL-1α, IL-1ß, IL-6, IL-10, IL-12, IL-33, IFN-γ and TNF-α) were measured by qPCR. All samples are compared with negative control (fold change = 1). Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

### 4.2.10 Recombinant IL-37a and IL-37b promote salmonella infection in murine macrophages

In order to understand if IL-37 is responsible for the difference of salmonella infection in human and murine macrophages, we next pre-treated the murine macrophages with rIL-37 before the salmonella infection, to see whether IL-37 is able to influence the course of salmonella infection. The RAW cells were cultured as before and treated with or without rIL-37a and b for 2 hours before the salmonella infection. Cell samples and culture supernatants were collected 6, 12 and 24 hours after infection. The bacteria CFU number, inflammatory gene level and NO production in each group were measured as before.

As seen in Figure 4.15b, the treatment of IL-37a, to a lesser extent IL-37b, significantly enhanced salmonella numbers in the infected murine macrophages 6 h after infection. At 12 hours, only IL-37a, not IL-37b, still enhanced bacteria numbers; at 24 h, no difference had been found in bacterial number between the IL-37a treated and untreated cells, while IL-37b significantly reduced the salmonella number. In general, the bacterial number in each group was reversely associated with the levels of iNOs and NO; as such, the untreated cells that carried less bacteria produced more NO (Figure 4.16a and b). in contrast, the IL-37 treated cells that contained more bacteria produced less NO. this phenomenon was even clear in the IL-37a treated cells (Figure 4.16a and b).

Furthermore, the inflammatory cytokines, IL-1α, IL-12 and IFN-γ levels in each of the experimental groups were also negatively associated with bacterial number, but positively associated with NO levels in most of the cases (Figure 4.17). This phenomenon was mimicked the salmonella infection in human cells. Thus, IL-37, particularly IL-37a, helps salmonella survival during its early infection in host macrophages.

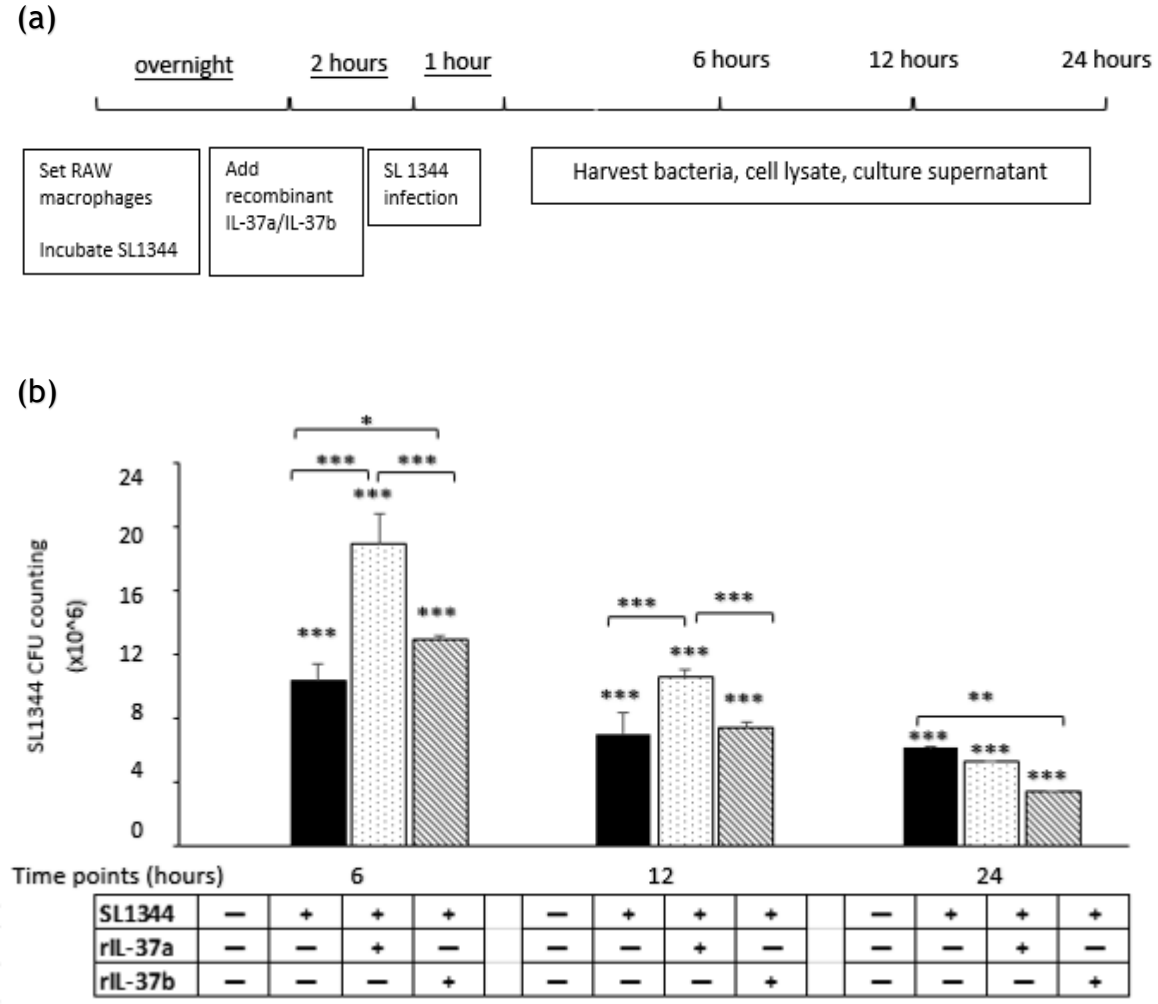



**Figure 4.15 Recombinant IL-37a and b proteins assist the survival of intracellular bacteria in murine macrophages**

Murine macrophage RAW cells (1x10^6 cell/ml) were pre-treated with 50ng/ml of mutant IL-37a and b proteins for 1 hour, then infected with SL1344 (1x10^8/ml) for 6, 12 and 24 hours. The cells were harvested and lysed for bacteria count on spot plates as before. a) Experimental and treatment processes. b) Bacteria counting result. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

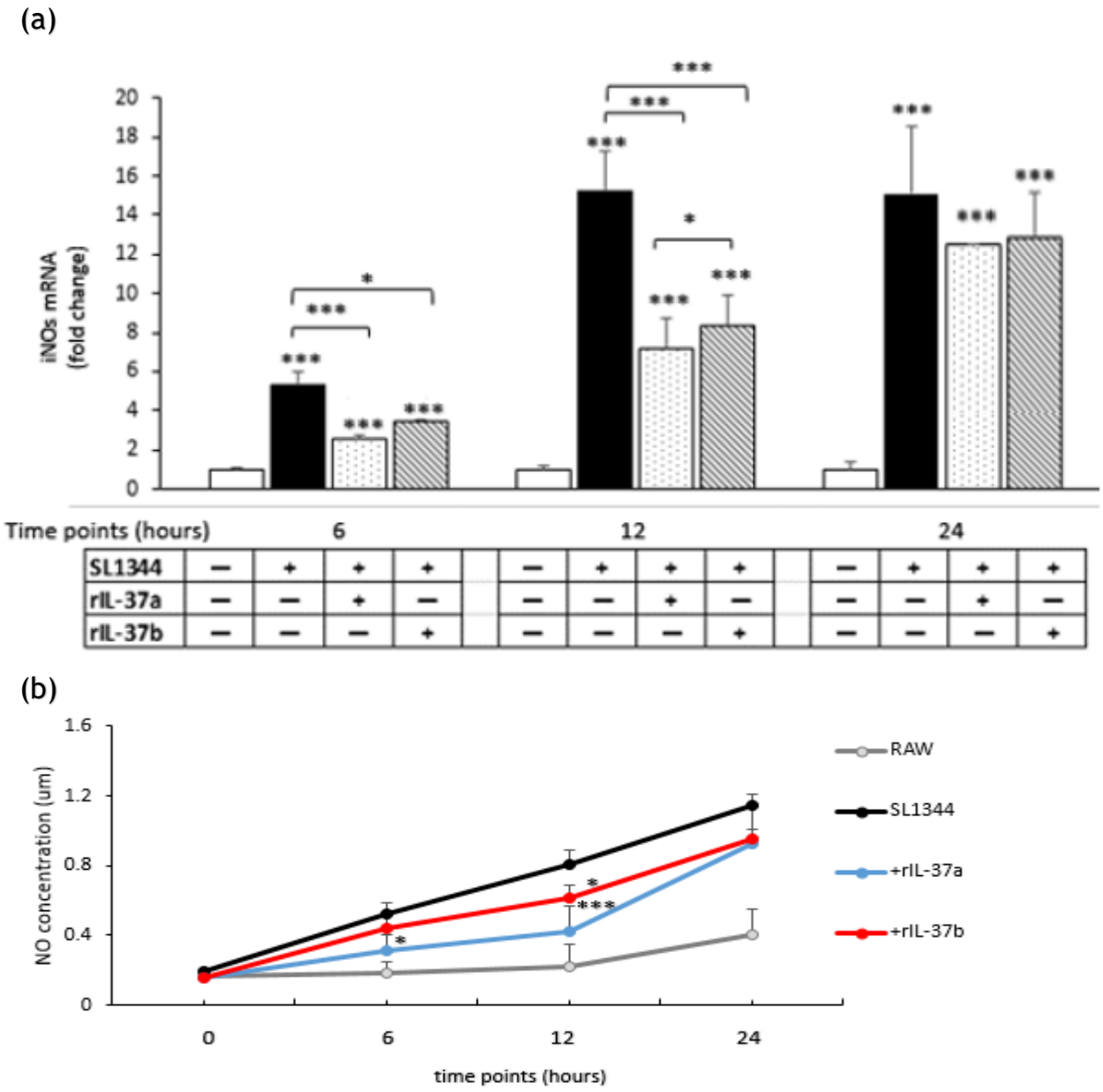



**Figure 4.16 Recombinant IL-37a and b proteins decrease the expression of iNOs and the production of NO in murine macrophage infected with Salmonella**

Murine macrophage RAW cells were pre-treated with 50ng mutant IL-37a and b proteins, then infected with SL1344 for 6, 12 and 24 hours. The cells were harvested and lysed for RNA extraction and cell culture supernatants were collected for NO detection. a) The kinetic of iNOs expression in infected RAW cells pre-treated with or without rIL-37a and b. b) NO production was measured by Greiss method. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, ***P<0.001 compared to controls.

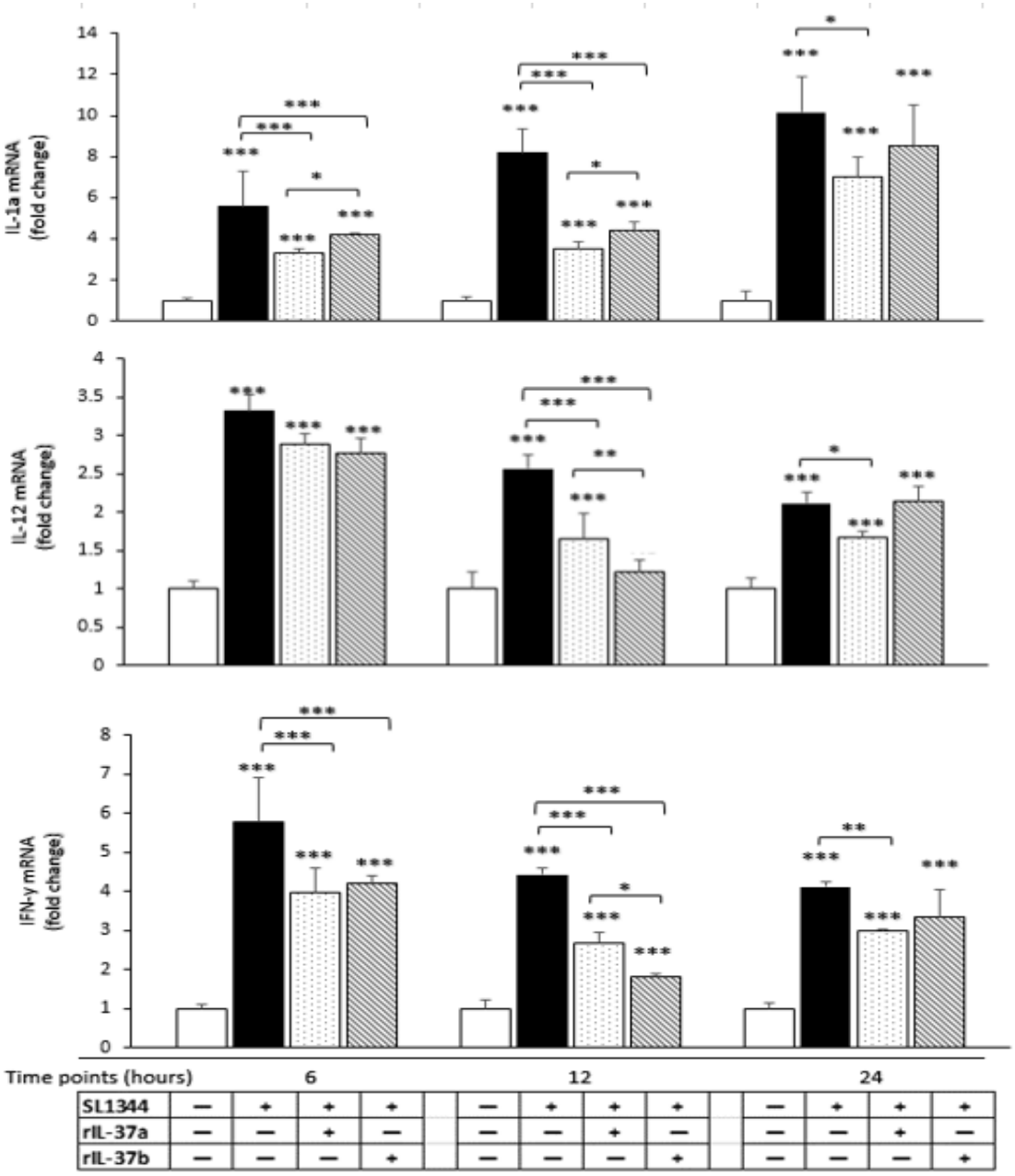



**Figure 4.17 Recombinant IL-37a and b proteins decrease the gene expression of pro-inflammatory cytokines in murine macrophage infected with Salmonella**

Murine macrophage RAW cells were pre-treated with 50ng each of mutant IL-37a or b proteins, then infected with SL1344 for 6, 12 and 24 hours. The cells were harvested for RNA isolation. The mRNA expression levels of four main pro-inflammatory cytokines (IL-1α, IL-6, IL-12 and IFN-γ) were measured by qPCR. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

### 4.2.12. Macrophages from IL-37aTg mice also susceptible to salmonella infection

After identifying the potential pathogenic role of IL-37 in salmonella infection in macrophage cell lines, we sought to confirm this finding in primary macrophages. peritoneal macrophages were collected from WT and IL-37aTg mice. These macrophages were infected with salmonella and the CFU were counted as before 6, 12 and 24 h after infection. the cells were also harvested for iNOs and inflammatory cytokine production by PCR and the cell supernatant for NO detection.

Consistent with the observations using macrophage cell lines and treated with recombinant IL-37, the peritoneal macrophages from IL-37atg mice also contained more intracellular bacteria than the WT cells at 6 and 12 hours after infection (Figure 4.18b). There was no significant difference in bacteria number between WT and Tg cells 24 hours after infection.

In agreement with our previous results in macrophage cell line, the enhanced bacterial number in cells from IL-37aTg mice was also accompanied by a significantly reduced levels of iNOs transcription and NO production in the cultures, compared to the WT cells (Figure 4.19a and b). This was also the case in the inflammatory cytokine expression levels (Figure 4.20).

This finding in primary macrophages further suggests that IL-37, particularly IL-37a helps early salmonella infection in macrophages, probably by inhibiting inflammatory cytokine and NO production.

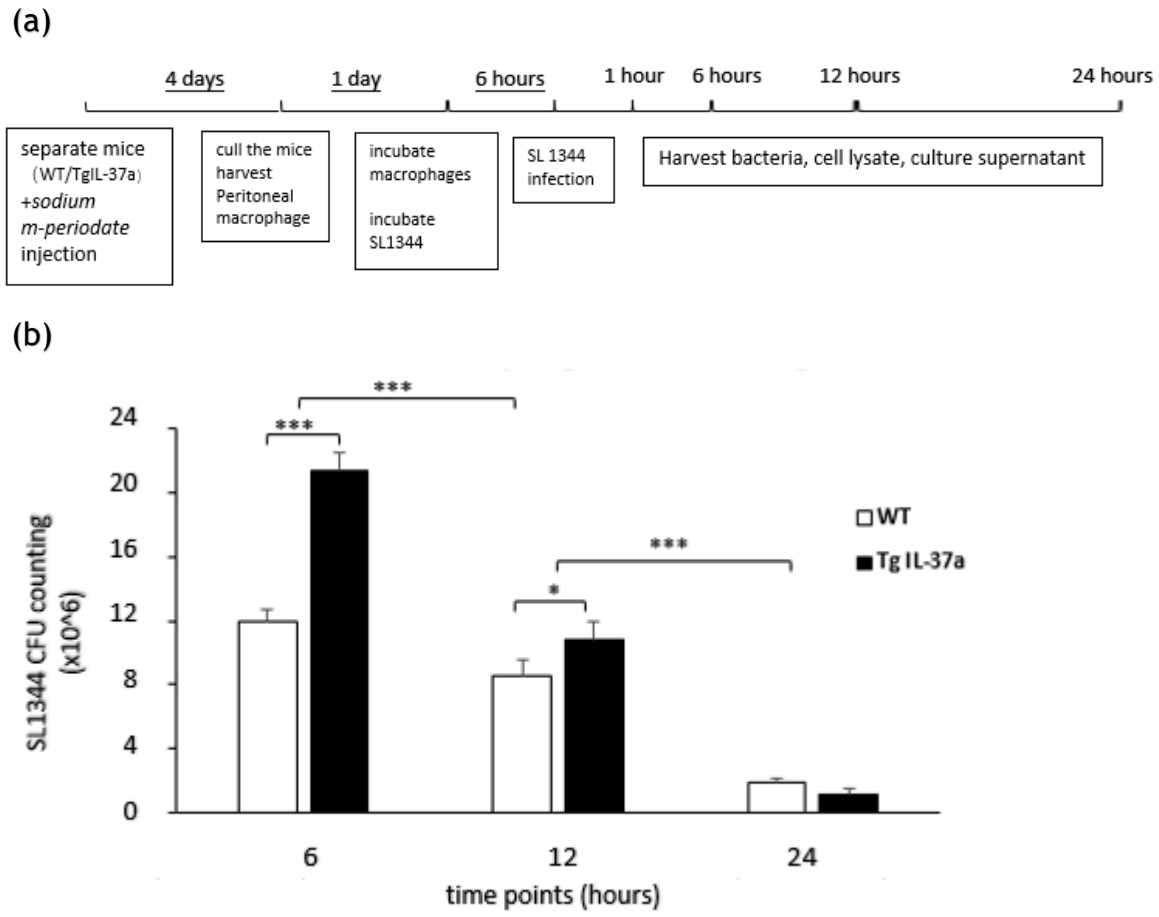



**Figure 4.18 The peritoneal macrophages from IL-37aTg mice had higher survival rate of intracellular bacteria compared with WT mice during Salmonella infection**

WT and IL-37aTg mice were injected peritoneally with sodium m-periodate. 4 days later, the peritoneal macrophages were isolated from mice and cells (1x10^6/ml) were infected with *salmonella* (1x10^8/ml) as before and collected 6, 12 and 24 hours after infection. The cells were harvested and lysed for bacteria count on spot plates. a) Experimental and treatment processes. b) Bacteria counting result. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05 and ***P<0.001 compared to controls.

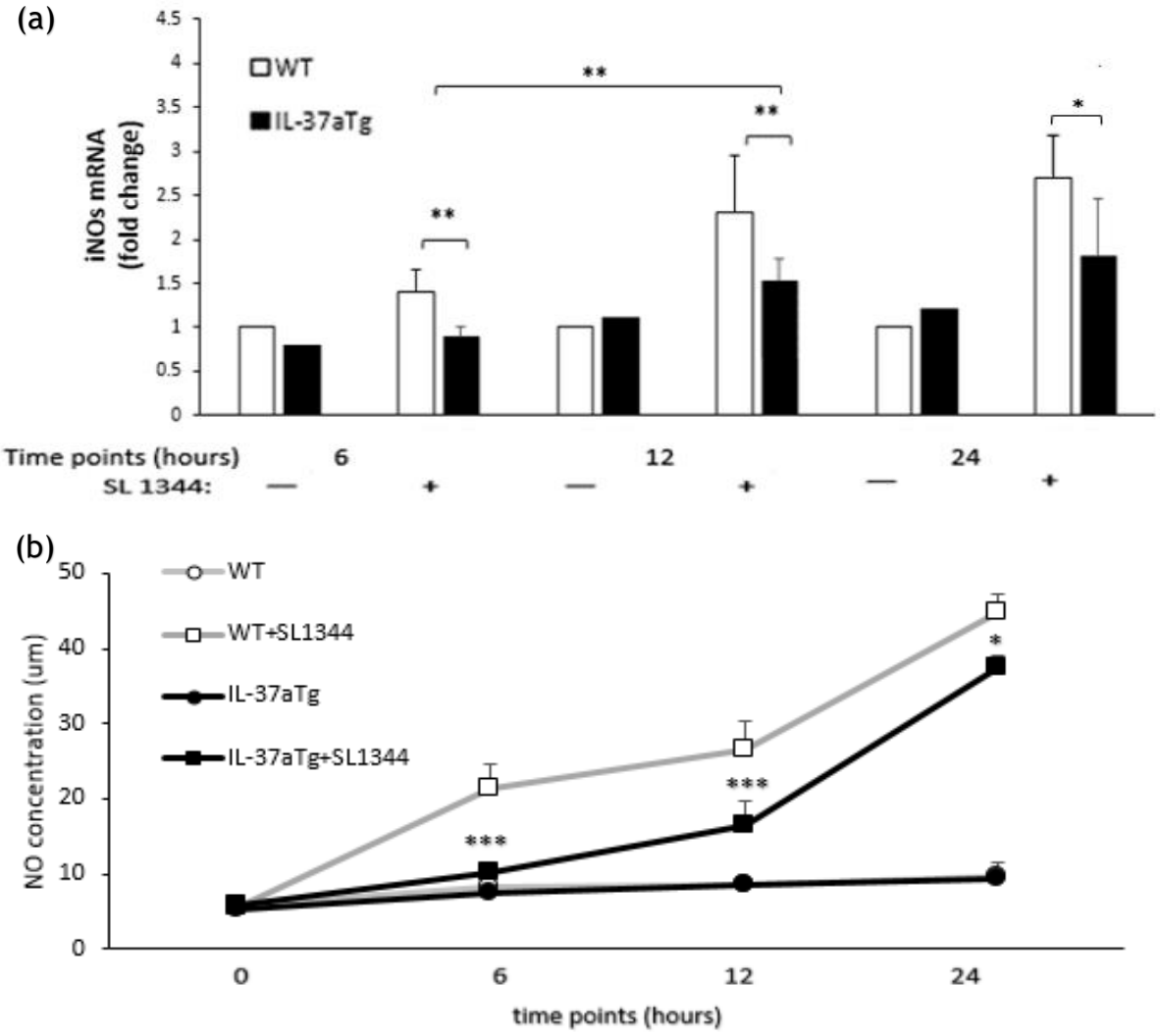



**Figure 4.19 The peritoneal macrophage from IL-37aTg mice reduce iNOs gene expression and NO production, compared with WT mice during salmonella infection**

Murine peritoneal macrophages were isolated from WT and IL-37atg mice injected with sodium m-periodate for 4 days. Cells (1x10^6 cell/ml) were washed and infected with salmonella (1x10^8/ml) for 6, 12 and 24 hours. The cells were harvested and lysed for RNA extraction and cell culture supernatants were collected for NO detection. a) The kinetics of iNOs gene expression in peritoneal macrophage of IL-37aTg and WT mice. b) NO production cells from WT and IL-37aTg mice. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, ***P<0.001 compared to controls.

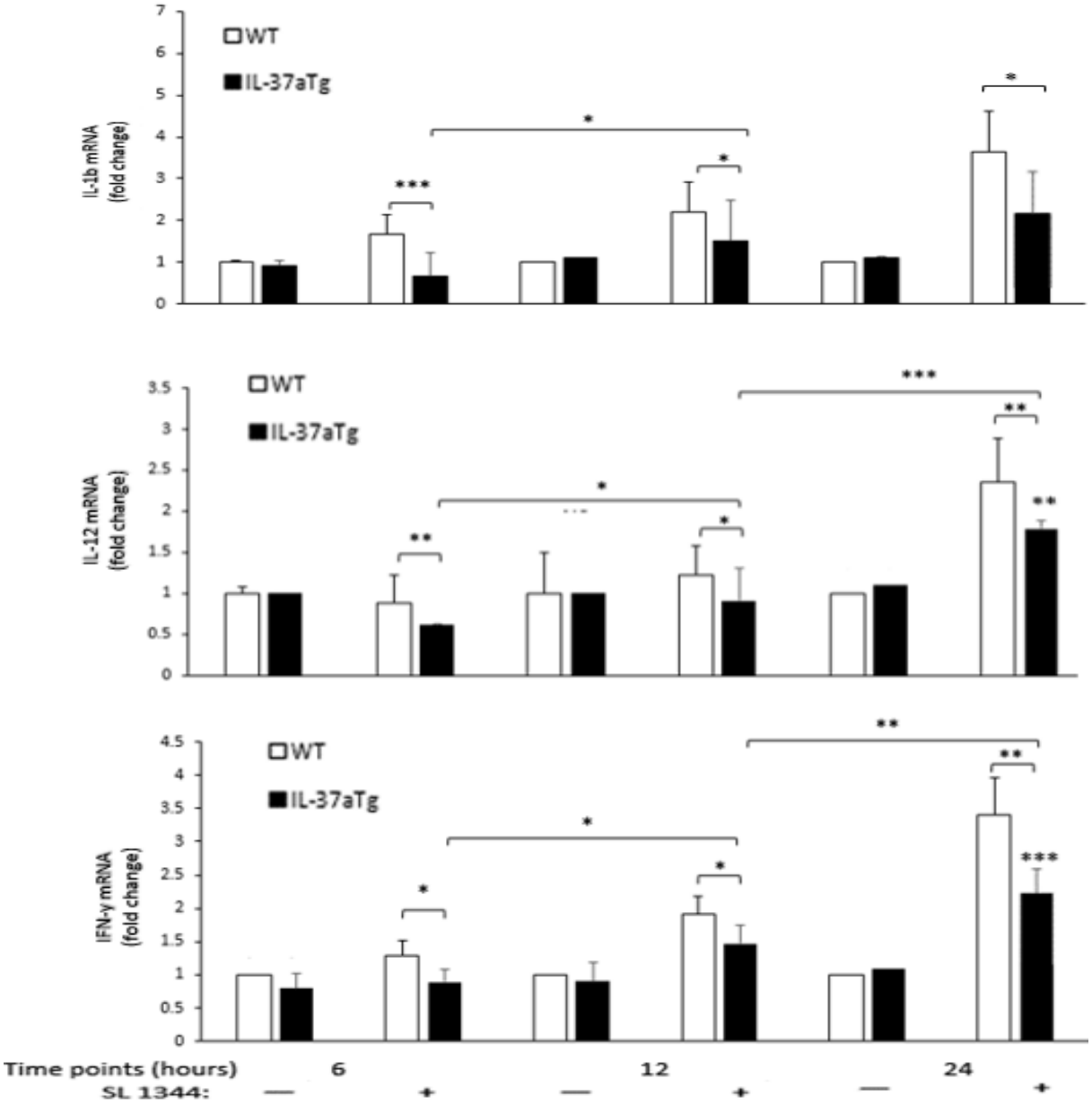



**Figure 4.20 The peritoneal macrophages from IL-37aTg mice express lower pro-inflammatory cytokine genes compared with cells from WT mice after salmonella infection**

Murine peritoneal macrophages were isolated from mice given sodium m-periodate stimulation for 4 days. Cells were washed and cultured in 1x10^6 cell/ml with *salmonella* (1x10^8/ml) for 6, 12 and 24 hours. The RNAs were extracted from total cell lysate and pro-inflammatory cytokine mRNA levels (IL-1ß, IL-6, IL-12 and IFN-γ) were measured by qPCR. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

### 4.2.13 IL-37aTg mice are susceptible to salmonella infection *in vivo*

Based on our results from the study of IL-37 in salmonella infection in macrophages *in vitro*, we thought it clinically important to further investigate the potential pathogenic role of IL-37 in salmonellosis in mice *in vivo*.

An attenuated salmonella strain BRD509 were used to infect the WT and IL-37atg mice orally by using our established method (Trieu *et al.*, 2009). Groups of WT and IL-37aTg mice were feed with 1x10^10 BRD509 orally. Their wellbeing and severity of diarrhoea in each group of mice were checked daily and recorded. Some mice from each group were culled at day 6 and day 8 after infection and splenocytes were collected for qPCR measurement, serum for NO detection and liver and drain lymph node for bacteria count. Firstly, the diarrhoea in IL-37a mice was much severe than WT at both 6 and 8 days after infection (Figure 4.21b). The IL-37aTg mice also had higher bacterial loading in both liver and LN at day 6 of infection, compared to the WT control mice (Figure 4.22). The difference was more pronounced at day 8.

Salmonella infection also enhanced the iNOs expression in spleen and serum NO production in both IL-37aTg and WT controls (Figure 4.23). However, the iNOs and NO levels in IL-37aTg mice were significantly lower compared to the WT control at both day 6 and 10 after infection.

Finally, the transcription levels of key inflammatory cytokines IL-1α, IL-6, IL-12 and IFN-γ in the infected and uninfected WT and Tg mice were also quantified by qPCR. The expression of all tested inflammatory cytokines in the BRD509 infected IL-37ATg mice were dramatically decreased in comparison with the WT mice at day 6 and day 8. This is consistent with the *in vitro* results using rIL-37 protein in salmonella infected macrophages. Therefore, the results demonstrated that IL-37a plays a critical pathogenic role in salmonella infection, that may facilitate intracellular survival in macrophages via downregulating protective inflammatory cytokine and bactericide NO production.

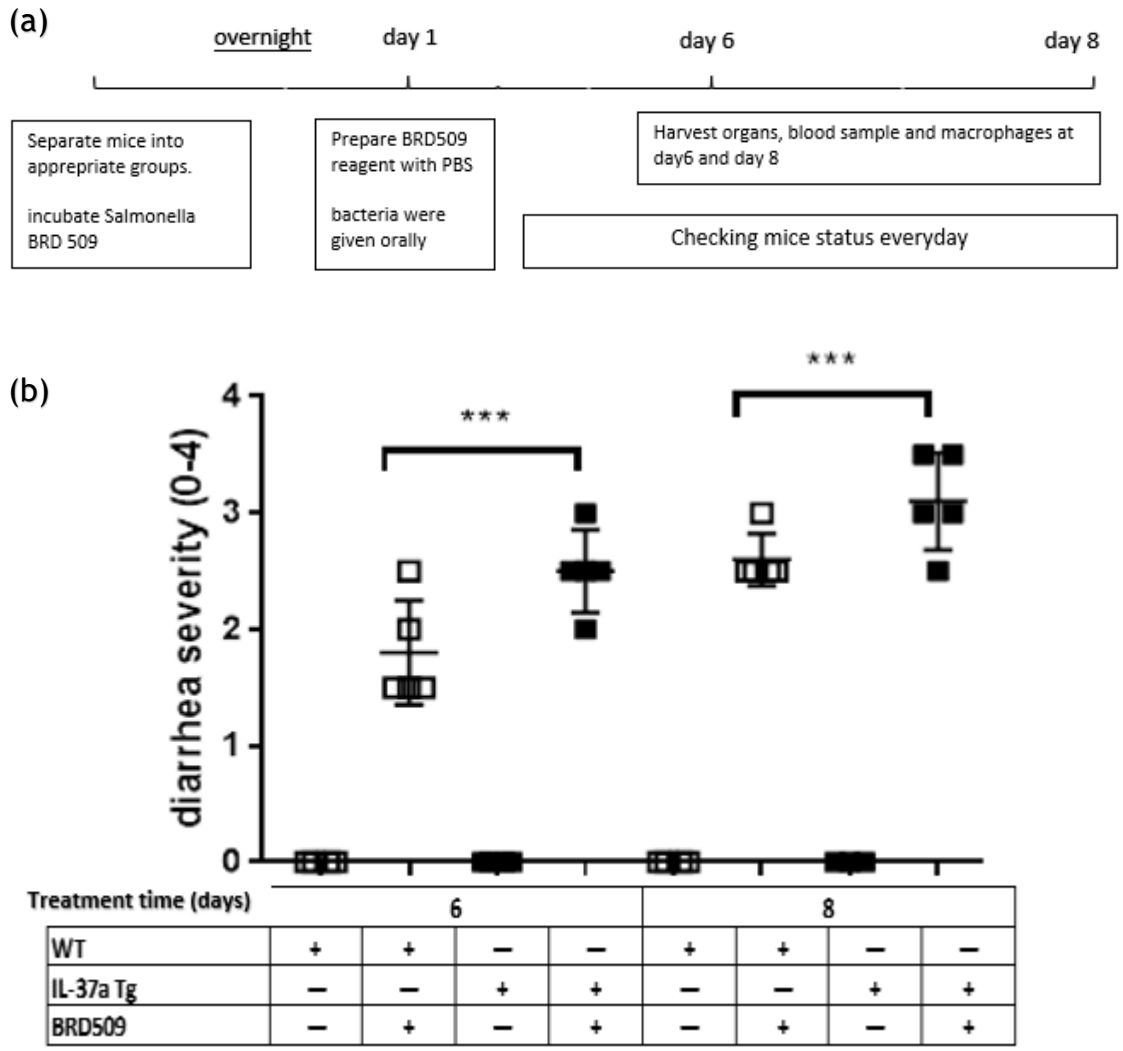



**Figure 4.21 Experimental process and diarrhoea severity of IL-37aTg mice and WT mice orally infected with salmonella**

IL-37aTg and WT mice were infected with *salmonella* strain BRD509 (1x10^10/mice) orally. a) Experimental and treatment processes. b) The diarrhoea severity of each mouse was observed at day 6 and 8 and recorded. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

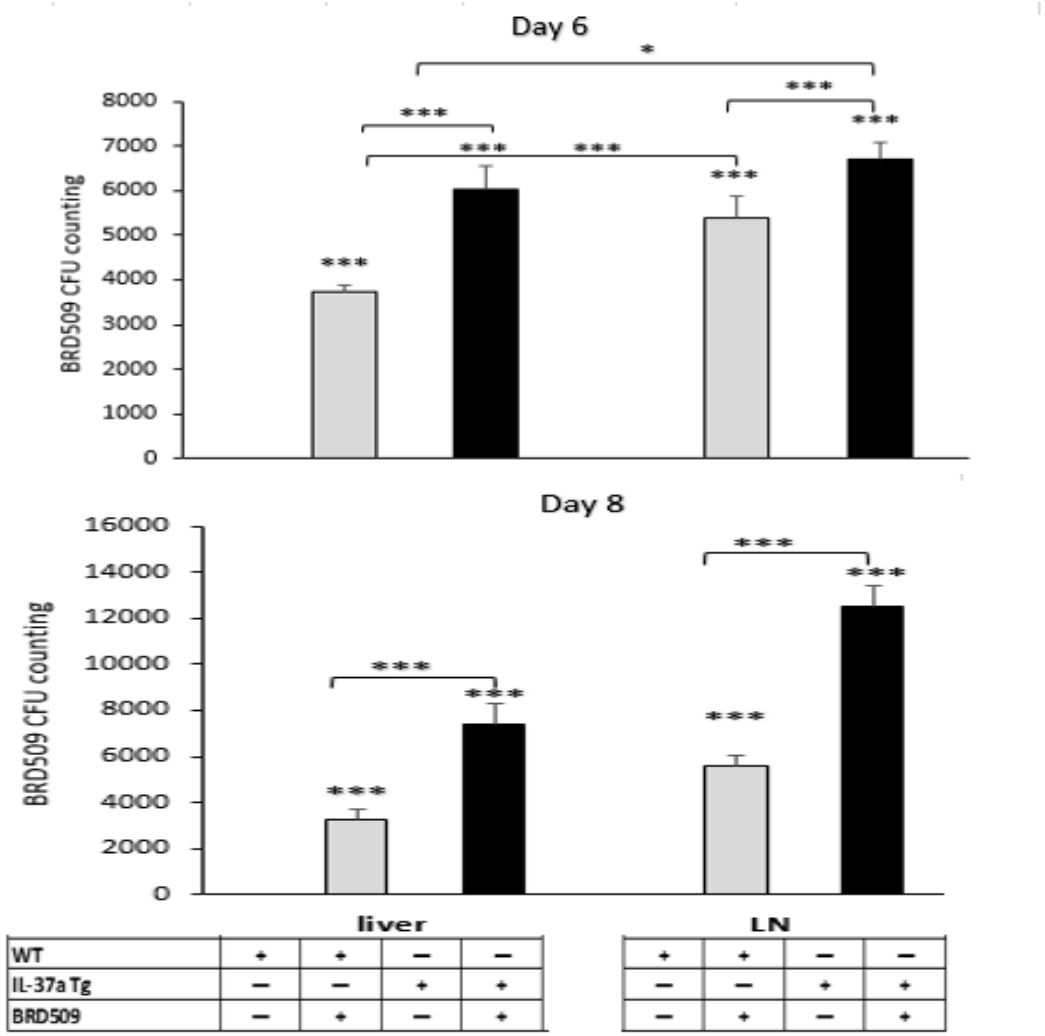



**Figure 4.22 Bacterial loading in organs of IL-37aTg mice and WT mice after BRD509 infection**

IL-37aTg and WT mice were infected with *salmonella* strain BRD509 (1x10^10/mice) orally. liver, and lymph nodes were collected on day 6 and 8 and bacteria number in the organs determined by spot plates. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

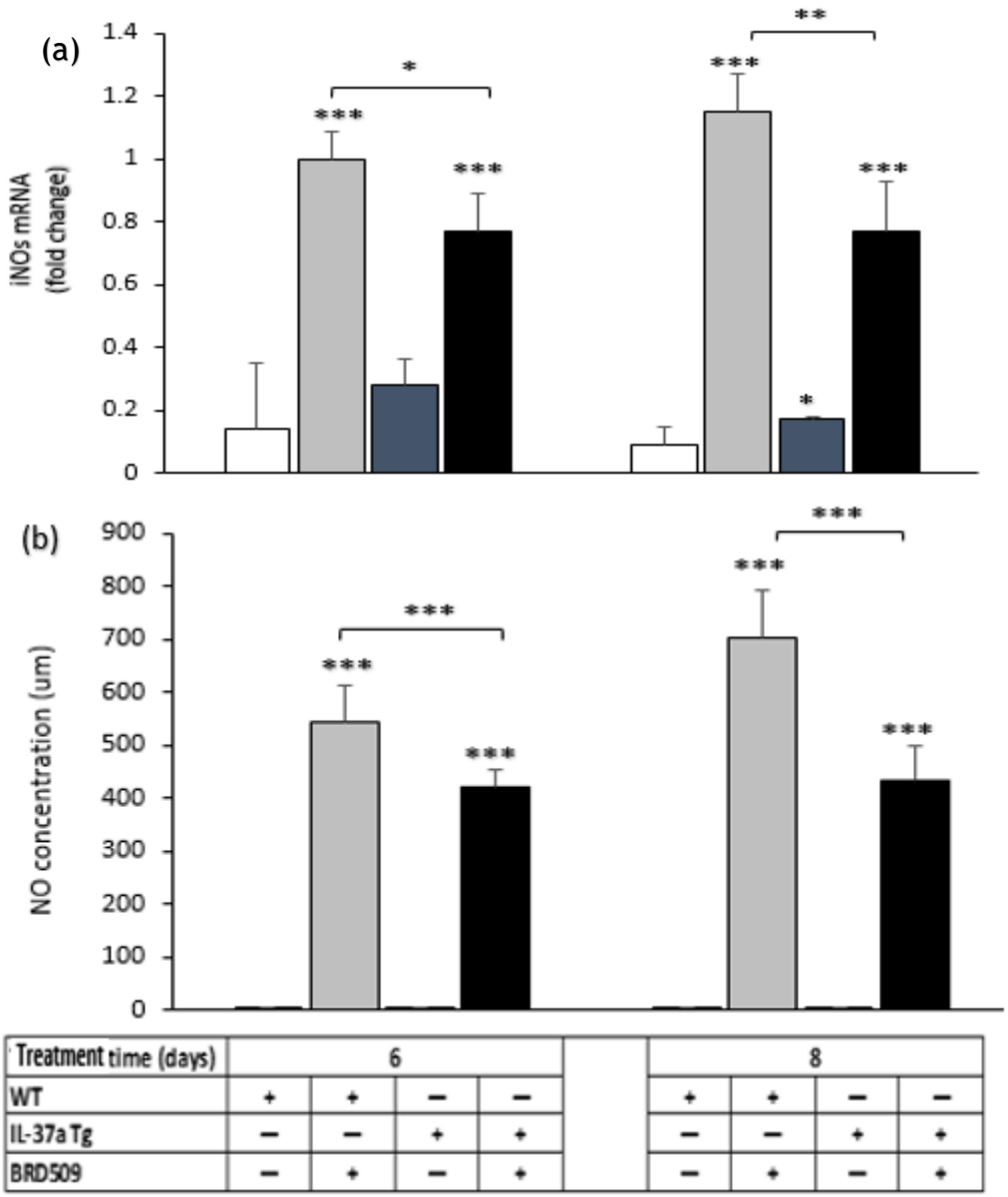



**Figure 4.23 Salmonella infected IL-37aTg mice have lower iNOs expression and NO production compared with WT mice**

IL-37aTg and WT mice were infected with *salmonella* strain BRD509 (1x10^10/mice) orally. Serum and spleens were collected from the infected mice. a) RNAs were extracted from total solenocytes for iNOs expression by qPCR; b) NO concentration in serum was detected by Griss kit. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

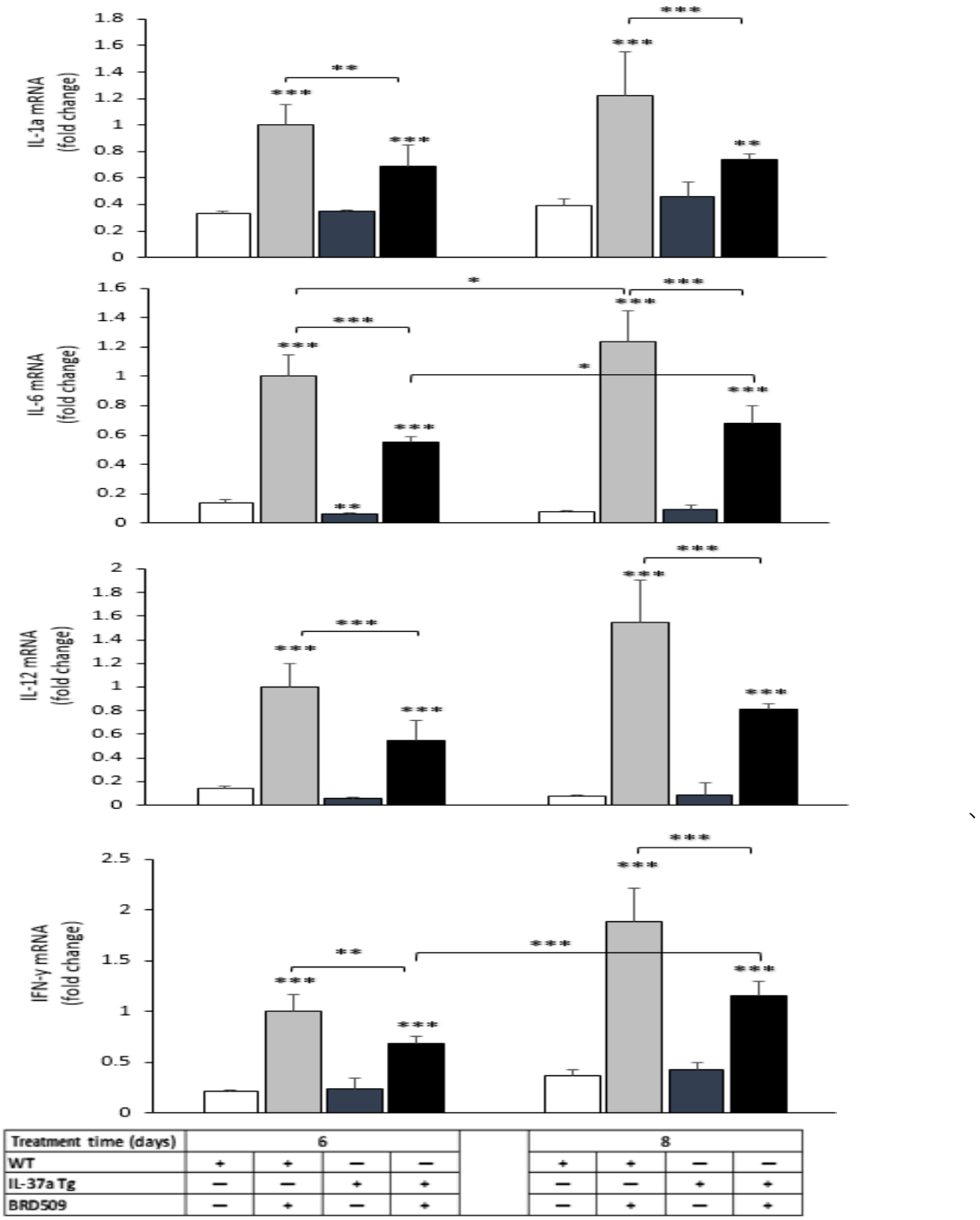



**Figure 4.24 IL-37aTg mice develop impaired pro-inflammatory cytokine mRNA levels after salmonella infection**

IL-37aTg and WT mice were infected with Salmonella strain BRD509 (1x10^10/mice) orally. RNAs were extracted from total splenocyte lysate, the mRNA level of pro-inflammatory cytokines (IL-1α, IL-6, IL-12 and IFN-γ) were measured by qPCR. Using ANOVA test, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

## 4.3 Discussion

In summary, I demonstrated in this part of the work for the first time that:

A) IL-37a is highly induced by TLR signals in human macrophages among the IL-37 isoforms. B) both endogenous and exogenous IL-37a are bioactive and can suppress TLR-induced inflammatory gene expressions. C) monomer form of IL-37a is better than the dimer in immunosuppression. D) IL-37a is more effective than IL-37b in the suppression of TLR response *in vitro*. E) IL-37a signals via receptor IL-1R8. F) human macrophages are more susceptible than murine cells and this may be due to their difference in IL-37 expression. G) IL-37a suppresses bactericide mechanism and facilitates salmonella infection in human macrophages *in vitro* and in mice salmonellosis. These findings suggest that IL-37a is a new immunoregulatory cytokine that suppresses TLR-induced inflammatory response, but, on the other hand, may also promote bacterial infection if dysregulated.

It is still unclear if IL-37 isoforms are equally or differently induced in a given immune context. To answer this question, we performed the experiment of time and dose-dependent induction of IL-37 isoform expression in TLR-stimulated human macrophages. The results demonstrated that IL-37 isoforms are differently regulated by different TLR signals and IL-37a is the most inducible one among the 5 isoforms in all tested TLR contexts. However, it is worthwhile to further investigate the expression pattern of IL-37a in other immune contexts, particularly in diseases.

While the precise mechanism by which TLRs regulate IL-37 isoform expression is still largely unknown, it has been suggested that IL-37 gene exon 5 encodes an instability element which can be stabilised by inflammatory signals (Boraschi *et al.*, 2011). Thus, TLR-inducing IL-37 expression may be, at least in part, via enhanced IL-37 mRNA stability. However, it is unknown why IL-37 isoforms are differentially regulated and why IL-37a is dominantly induced.

Until now, it is still unknown whether IL-37a is bioactive. Using several approaches, including siRNA, recombinant protein and transgene, we demonstrated that IL-37a is bioactive and can suppress TLR-induced inflammatory response. In identifying IL-37a transgenic mice, we used both ELISA and qPCR. Because ELISA can only measure secreted IL37 and doesn't account for any IL37 in the nucleus. Although we detected the IL-37a level in transgenic mice serum by ELISA already, there is still necessary to check the mRNA expression by qPCR. We (me and others in the group) found that IL-37a is able to inhibit the function of all known TLRs located on cell surface or intracellular (this chapter and unpublished results). Thus, IL-37a is likely to be an important regulatory factor for TLR response. The regulatory effect of IL-37a that we found so far is exclusively immunosuppressive.

Intriguingly, we found that rFLIL-37a is more suppressive than rFLIL-37b in TLR response. Given that IL-37a is the most induced among the 5 isoforms in all tested TLR contexts, also the knockdown total IL-37 and IL-37a had similar degree of enhancement of TLR response in macrophages, together, these results suggest that IL-37a may be a dominant isoform and responsible for most IL-37-mediated immunoregulatory effects, at least, TLR-induced inflammatory responses. However, it is essential to further study the induction and function of IL-37a in other immune contexts, particularly in diseases, for instance, autoimmunity, cancer and allergy.

Using IL-1R8KO mice, we defined that IL-1R8 is an IL-37a receptor. This suggests that IL-1R8 is a receptor for both IL-37a and b. The IL-37 receptor complex consists of IL-1R8 and IL-18a and both chains are required for IL-37 signalling (Nold-Petry *et al.*, 2015). IL-18Ra is involved in the initial interaction with IL-37 protein, that then subsequently recruits IL-1R8 for signalling. While we have not determined the association of IL-18Ra in IL-37a signalling, it is most likely that it will be involved in IL-37a signalling. We found that IL-18Ra is highly induced compared to IL-18R by IL-37a in macrophage. The importance of IL-18Ra in IL-37a signalling will be investigated by siRNA knockdown or blocking antibody.

It is still unknown why rFLIL-37a is superior to rFLIL-37b in activity. Since we found that mature IL-37a has comparable bioactivity with mature IL-37b (data not shown), it is possible that their difference in bioactivity may be attributed to their difference

in N-terminal sequence. Since both isoforms bind to the same receptor, the different N-terminal sequence may differently influence their receptor binding affinity and signalling. Testing their relative binding affinity with receptor may answer the question. Nevertheless, this further suggests that IL-37a is a major cytokine in all IL-37 isoforms.

We found that same as IL-37b, IL-37a monomer is also more effective in suppressive function than its dimer. While the reason is still less understood, it is possible that the dimer may interfere its interaction with IL-37 receptor and signalling. The monomer form IL-37a may be more useful in research and potential clinical application in the future. Interestingly, the high dose of IL-37 dimers not only fails to suppress TLR response but enhances the response. Again, the reason behind is unclear and deserves more investigations.

TLR, in particular TLR2, 4 and 5 signals play a critical role in salmonella infection. After identifying the regulatory effect of IL-37a on TLR, including the salmonella related TLRs, TLR2, 4 and 5, we explored the potential importance of IL-37a in salmonella infection by developing several *in vitro* and *in vivo* experiments and found that IL-37a downregulates host protective immunity and bactericidal mechanism, and thereby promotes salmonella infection.

Firstly, we found that salmonella infection primarily enhanced IL-37a expression in human macrophages. These consistent results shown that IL-37a is also highly induced among the IL-37 isoforms by TLRs, particularly TLR4 in macrophages. In order to understand the physiological and pathological meaning of the selective expression of IL-37 in human but not in mice in infection, we systemically compared the ability of murine with human macrophages in clearance of salmonella infection. Our results suggest that human macrophages are less effective in the clearance of salmonella bacteria compared to that in murine cells. To understand if IL-37 is responsible for the deficiency of salmonella infection in human, we further determined the role of IL-37a in salmonella infection in mice *in vitro* and *in vivo* using recombinant and transgenic IL-37a. These results clearly demonstrated the IL-37a is able to promote salmonella infection in the early stage.

While the detailed mechanism underlying IL-37a promoting salmonella infection is still not yet fully understood, our preliminary results from salmonella infected macrophages or organs *in vitro* and *in vivo* suggest that IL-37a may do so by suppressing the key bactericidal mechanism-NO production in macrophages; we demonstrated that after salmonella infection, human macrophages which express IL37 produce lower levels of NO than murine cells that do not express IL-37. Since IL-37a also suppresses iNOs expression it is possible that the reduced NO production in infected macrophages was attributed from the reduced iNOs transcription.

However, the IL-37a-reduced NO in salmonella infection is also accompanied by reduced inflammatory cytokine production. It is well known that iNOs can be induced by inflammatory cytokines, including IL-1, IL-6 and IFN-γ (Jin *et al.,* 2016). Thus, the reduced iNOs/NO may also be the consequence of IL-37a inhibited inflammatory cytokine production in the salmonella infected macrophages. Further studies are needed to define the direct or indirect effect of IL-37a in iNOs/NO production and also the physiology or pathology of IL-37a in salmonella infection.

Th1 cells play a protective role against salmonella infection (Pashine *et al.*, 1999). Since this study was focused on the role of IL-37a in the regulation of innate immune response in early salmonella infection in macrophages the potential importance for IL-37a in the modulation of Th1 function in salmonella infection has not been explored. However, we found that IL-37a is able to suppress the Th1 cytokines production in macrophages *in vitro* and in splenocytes *in vivo*, thus, it may be expected that IL-37a may be also capable of inhibiting Th1 cell development and function in salmonella infection.

Based on current knowledge and our findings, we would like to propose a possible role and mechanism of IL-37a in salmonella infection: During salmonella infection, the bacteria will be phagocyted, internalised and amplified in the phagosomes of macrophages. The salmonella related TLR agonists, LPS, BLP and flagellins will be reorganised by the TLRs on the macrophages and induce inflammatory cytokine and NO production to kill the intracellular bacteria. In the meantime, salmonella related

TLR agonist will also induce IL-37, in particularly IL-37a expression that has feedback effect to inhibit TLR-induced inflammatory cytokine and NO-mediated inflammation and bactericidal, thereby facilitating salmonella infection by inhibiting innate immune response.

There are still some shortages which need to be developed in the future experiments:

In this chapter, we illustrate that the existence of IL-37, especially IL-37a may be an explanation why murine and human macrophages act differently in salmonella infection and TLR stimulations. However, the differences between human and mice are not only in IL-37 expression. There are other factors which may affect their sensitivity in inflammation and infection disease:1) In humans, the type I IFN, IFN-α which secreted macrophages, acts on T cells to induce Th1 development. This process is dependent upon STAT4 activation. In mice, however, IFN-α fails to induce Th1 cells and does not activate STAT4 (Farrar *et al.*, 2000). 2). The expression of iNOS in mouse macrophages has been clearly demonstrated and iNOS mRNA can be highly induced by LPS. However, these same inflammatory mediators have failed to show consistent effects on human macrophages. Instead, other mediators, such as IFN-a/b and various chemokines work more efficient in inducing iNOS in human macrophages (Bogdan, 2001). This phenomenon may also explain why human macrophage has lower iNOS expression in TLR stimulation condition.

Moreover, in this experiment, we use murine macrophage cell line RAW 264.7 cell compare with human macrophage cell line THP-1 in TLR stimulation and salmonella infection *in vitro*. The Raw cell are SV40 transformed peritoneal macrophage from a male BALB/c mouse. They are more sensitive than primary macrophage in stimulation and bacteria infection. In this case, the pro-inflammatory cytokine expression level would be higher than normal condition. To address this, we used murine peritoneal macrophage to test the infection condition *in vitro*. However, we did not use human primary macrophage as a control here to compare the difference between human and mice in salmonella infection, which need to be done in the early future.

Furthermore, the experiment condition can be further optimized. In my experiment, I test the mice condition on day6 and day8 in WT and IL-37aTg mice after infection. However, whether the bacteria number in IL-37aTg mice will continually increase or not remains a question. In the recovery stage, what is the function of IL-37a and how IL-37 be regulated is still unknown. Therefore, the experiment condition can be optimized by extend the infection time, in order to check in which time point IL-37 start their function and in the later stage of infection whether IL-37aTg mice have higher mortality than WT.

Although the pathophysiological meaning of the selective expression of IL-37 in human but not mice in salmonella infection is still unclear, our result is in agreement with the report that mice have higher rates of production of reactive oxygen species and suffer higher rates of oxidative damage than do humans (Kalghatgi *et al.,* 2013). Thus, IL-37a promoted salmonella infection may be accidently rather than deliberately. Furthermore, this finding may also provide explanations, at least in part, for why mice are more resistant to foodborne infections.

# Chapter 5

# Transcriptomic analysis of LPS-induced genes modulated by transgenic IL-37 in murine cells

# 5. Transcriptomic analysis of LPS-induced genes modulated by transgenic IL-37 in murine cells

## 5.1 Introduction

In Chapter 4, I have demonstrated that IL-37a is bioactive and able to suppress inflammatory cytokine expression induced by all tested TLR ligands in splenocytes, monocytes and macrophages. In addition, IL-37a and IL-37b display similarity and difference in biology and function. However, the molecular mechanism underlying their common and different in bioactivity is still not yet understood. In this chapter, using LPS/TLR4-induced inflammatory response in splenocyte as a model, I performed transcriptomic experiments to reveal the common and unique gene and signalling pathways regulated by IL-37a and/or IL-37b in LPS response. This part of work was also aimed to provide some molecular explanations for the regulatory role of IL-37a and b in the LPS/TLR4 signalling-mediated effect on macrophages and in salmonella infection in Chapter 4.

TLR4 signalling in innate immune cells, including macrophages, has been well studied and is described in Chapter 1. Briefly, TLR4 signals via a MyD88-dependent and TRIF-dependent pathway. The former is triggered by an agonist binds to the cell surface TLR4 complex, and the recruitment of MyD88 and adaptor TIRAP (MAL) and the activation of signalling cascades nuclear factor-kB (NF-kB) and mitogen-activated protein (MAP) kinases, resulting in inflammatory gene expression. The latter is elicited when the TLR4 is internalised into cell plasma and the recruitment of adaptor TRIF and TRAM, leading to the activation of interferon (IFN) response factors (IRFs) and subsequent type I IFN expression (Medzhitov, 2001; Wang *et al.*, 2001).

While the IL-37a signalling pathway is unknown, the IL-37b signal in LPS response has been studied (Nold-Petry *et al.*, 2015 and Chapter 1). In brief, the splenocyte RNA was isolated from wild-type and IL-37tg mice 3 hours after intraperitoneal injection of LPS, and the immune gene profiles were analysed by RNA-sequencing

along with the Innate Immune Database (Innate DB). The results indicate that 1) IL-37b not only suppresses but also enhanced gene expressions compared to the WT control, with the suppression effect dominant. 2) IL-37b primarily inhibited genes that are largely associated with inflammatory response, including inflammatory cytokines, chemokines, metabolism and signalling molecules. 3) IL-37b induced some genes with immunoregulatory property. (Nold-Petry *et al.*, 2015). This work has established IL-37b signalling and function in gene regulation. While how IL-37a signal regulates inflammatory genes is still largely unknown, the research in IL-37b signalling is helpful for the understanding of IL-37a signalling and the difference between the two isoforms. Based on all the results from our own and from other lab, I propose that: the functional similarity and difference between IL-37a and IL-37b be attributed to their similarity and difference in protein sequence and signalling transduction. Using their sheared C-terminal protein sequence containing IL-1-like domain for receptor binding and signalling, IL-37a and IL-37b may regulate common signalling pathways and genes. However, given their difference in N-terminal sequence, they may regulate different pathways and genes. The overall aim in this chapter was to test this hypothesis using LPS/TLR4 activated murine splenocytes from IL-37a and b transgenic mice as a cellular model to understand how IL-37a and IL-37b regulate TLR signalling transduction and what are the common and difference between the two IL-37 isoforms in gene regulation.

The aims of the project were to:

1. Identify the global different expressed genes regulated by IL-37a and IL-37b with RNA-sequencing and bioinformatics assay.

2. Assess the immune genes/signalling pathways which are commonly regulated by both IL-37a and IL-37b.

3. Identify the immune gene/pathways specifically regulated by IL-37a or IL-37b.

4. Explore the possible molecular mechanism by which IL-37a and IL-37b regulate LPS/TLR4-mediated inflammatory response.

## 5.2 Results

### 5.2.1 Identifying the early LPS-induced genes differentially regulated by IL-37a and IL-37b transgenic cells

To explore the common and specific regulatory effect of IL-37a and b on TLR-stimulated gene expression, I systematically identified and compared genes in splenocytes of IL-37a and b transgenic and WT mice using transcriptome analysis. To be more specific, splenocytes from IL-37a and b transgenic and WT control mice were seeded in 24 wells cell culture plate in the density of 1x10^6/ml. These cells were stimulated with 500 ng/ml LPS for 5 hours. Total RNA from each sample was isolated, cDNA libraries were constructed, and the next-generation sequencing performed using RNA-sequencing. The results were analysed by bioinformatics which mentioned in Chapter 2.

To understand the differentially expressed genes regulated by IL-37 a and b, the global differentially expressed genes (DEG) between LPS- stimulated splenocytes from WT and IL-37a and b transgenic mice were initially identified. Raw data from RNA-seq were analysed for each group (WT, IL-37a and IL-37b transgenic mice, n=3). The average RPKM (Reads Per Kilobase Million) per group was estimated. All results from IL-37a and IL-37b groups were compared with the WT control. Upregulated genes and pathways mean compare with control group, the same gene expression enhanced by the existence of IL-37a or b and the pathway they belong to. Downregulated genes and pathways mean compare with control group, the same gene expression decreased by the existence of IL-37a or b and their pathways. The cut off p value ($< 0.05$) and fold-change ($\geq 1.5$) were used to detect the differential expressed genes between IL-37a or b transgenic and WT cells.

Firstly, the global DEG between LPS-stimulated cells between IL-37aTg and WT mice were identified, including the upregulated and downregulated genes using bio Venn programme and visualised by the area-proportional and overlapping Euler (Figure 5.1). As shown in the Euler diagrams, compared with WT control (2147 genes), 1511 from the total analysed genes were significantly downregulated by IL-37a (Figure 5.1a). 1068 genes also upregulated by IL-37a compared with the WT control (Figure 5.1b).

With the same analysis strategy, the global DEG between IL-37b and the WT control were also identified (Figure 5.2). In comparison with the WT control, there were 1849 genes downregulated by IL-37b (Figure 5.2a). Furthermore, 1321 genes were identified which were upregulated by IL-37b (Figure 5.2b).

Next, the commonly and specifically regulated genes by IL-37a and b were further identified by comparing the DEG of IL-37a and b gene pools. As shown in Figure 5.3, the downregulated DEG between IL-37a and b groups were compared and illustrated by Venn diagram. Among all these genes, 738 DEG were the common genes downregulated by both IL-37a and b (about 28% of common/total genes); 773 genes were detected to be specifically downregulated by IL-37a and 1111 genes were specifically downregulated by IL-37b (Figure 5.3a).

Furthermore, by comparing the upregulated DEG of IL-37a and IL-37b groups, 336 genes were found to be commonly upregulated by these two IL-37 isoforms (the rate of common gene is 16%, (common/total genes); 732 genes were detected to be specifically downregulated by IL-37a and 985 genes were specifically downregulate by IL-37b (Figure 5.3b).

Thus, IL-37a and b not only suppressed but also enhanced the expression of some LPS-induced genes. Our result also suggests that IL-37b may regulate more genes compared with IL-37a in this context, 1.17 folds more downregulated genes and 1.23 folds more upregulated genes.

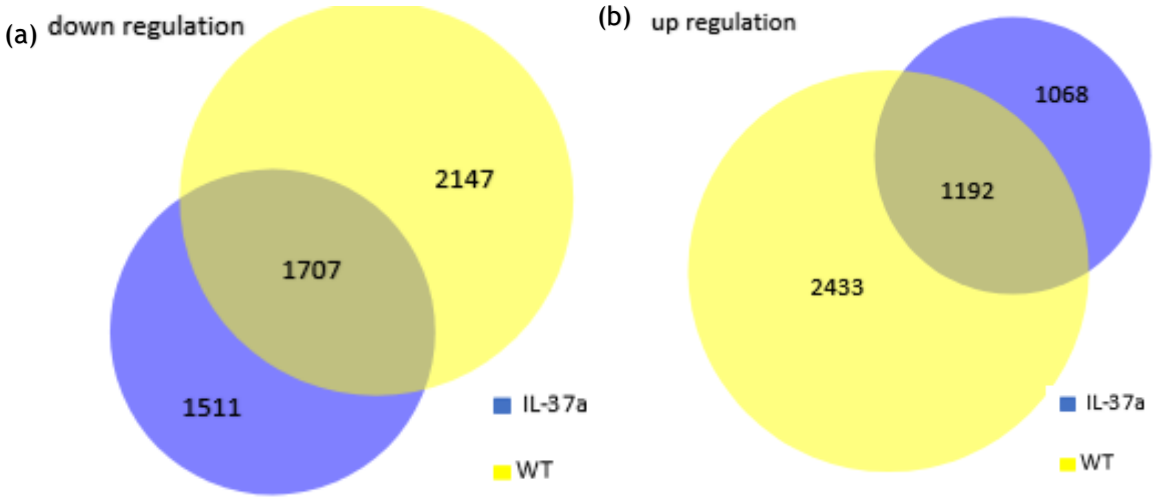



**Figure 5.1. Identification of the differentially expressed genes regulated by IL-37a in LPS stimulated transgenic mice splenocytes compared with WT**

Splenocytes from IL-37aTg mice were compared with WT after 500ng/ml LPS stimulation for 5 hours; total RNAs from each group were isolated for microarray. The differential expressed genes identified using bio Venn programme and visualised by the area-proportional and overlapping (relationship) Euler. a) shows differentially expressed genes (DEGs) that are specifically downregulated by IL-37a (blue area) compared with WT control; the rest of yellow area are genes not affected by IL-37a. b) The blue area is DEGs that are specifically upregulated by IL-37a; the overlap area are the common genes both up regulated in IL-37a and WT; while the rest of yellow area are genes not influenced by IL-37a.

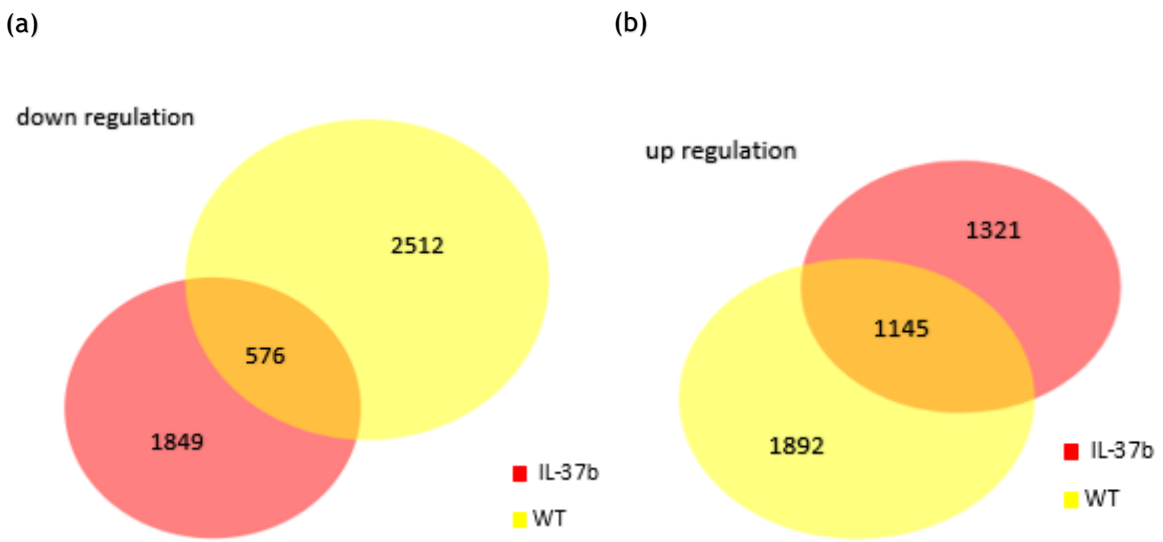



**Figure 5.2. Identification of the differentially expressed genes regulated by IL-37b in LPS stimulated transgenic mice splenocytes compared with WT**

Splenocytes from IL-37bTg mice were compared with WT after 500ng/ml LPS stimulation for 5 hours; total RNAs from each group were isolated for microarray. The differential expressed genes identified using bio Venn programme and visualised by the area-proportional and overlapping (relationship) Euler. a). Differentially expressed genes (DEGs) that are specifically downregulated by IL-37b (red area) compared with WT control; the rest of yellow area are genes not affected by IL-37b. b). The red area is DEGs that are specifically upregulated by IL-37b; the overlap area are the common genes both up regulate in IL-37b and WT; while the rest of yellow area are genes not influenced by IL-37b.

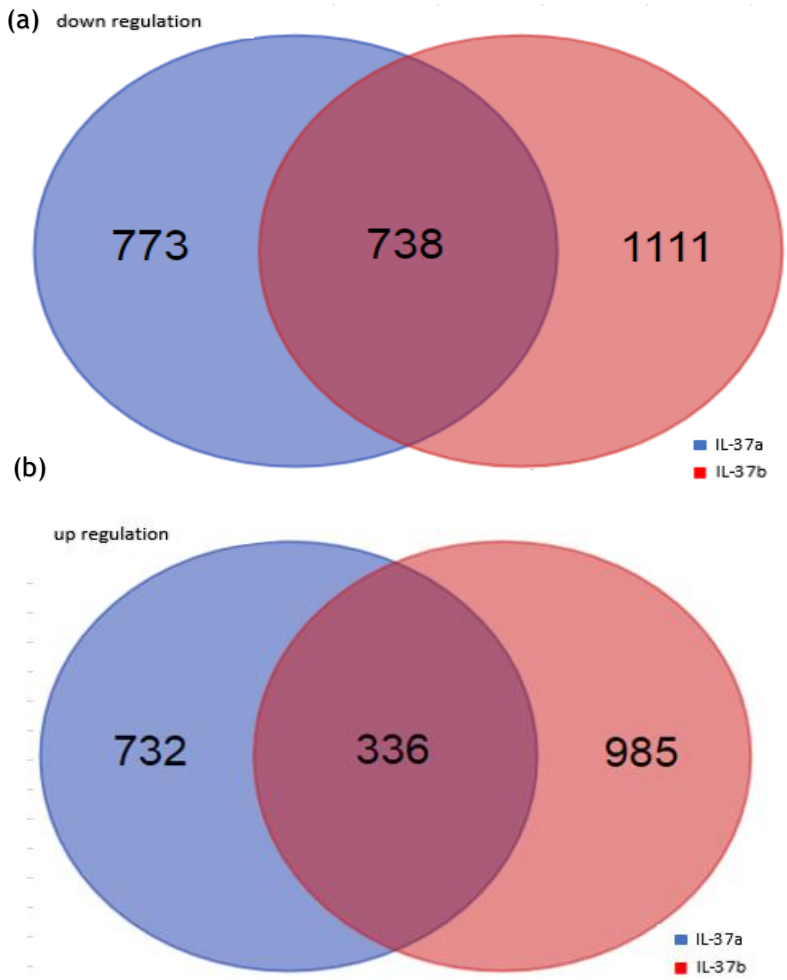



**Figure 5.3 Identification of common and specific genes differentially regulated by IL-37a and b in LPS stimulated transgenic mice splenocytes**

Splenocytes from IL-37a and IL-37bTg mice were compared with each other after 500ng/ml LPS stimulation for 5 hours; total RNAs from each group were isolated for microarray. The differential expressed genes identified using bio Venn programme and visualised by the area-proportional and overlapping (relationship) Euler. a) represents the genes that are downregulated by IL-37a and b; the red area shows genes downregulated by IL-37a only; the overlap area shows the genes that are downregulated by both IL-37a and b; while the blue area represents the genes that are downregulated by IL-37b only. b) shows the set of genes that are upregulated by IL-37a and b; specific genes upregulated by IL-37a shown in red area; the overlap area are common genes, which are upregulated by both IL-37a and b; the blue area are genes that are upregulated specifically by IL-37b.

### 5.2.2 Revealing the common signalling pathways/genes regulated by both IL-37a and IL-37b

To explore the molecular mechanism by which IL-37a and b regulate LPS/TLR4 response in TLR stimulated immune cells *in vitro* and salmonella infection in vivo, I further identified the nature of the common and specific genes regulated by IL-37a and b and related signalling pathways. The Innate Immune Database (Innate DB) was used as a general immune gene research database in our research, which was generated from several major databases and focused on, but not limited to the innate immunity by curating with experimentally-validated human and mouse interactions from the biomedical literature (Lynn *et al.*, 2010). It provides a manually curated knowledgebase of the genes, proteins, and particularly, the interactions and signalling pathways involved in mammalian immune response, including TLR-mediated response (Lynn *et al.*, 2010; Breuer *et al.*, 2012). Based on our research direction and the research results in Chapter 4, the TLR signalling and Salmonella infection relate pathways are particularly focused on and studied in more details.

Firstly, gene ontology of these common genes, 738 downregulated and 336 upregulated (Figure 5.3) by both IL-37a and IL-37b were identified using Innate DB and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. From these analyses, 40 signalling pathways were significantly downregulated by both IL-37a and b (data not shown), 7 of 40 pathways which related to our research aims were selected and shown in Figure 5.4a, including T cell receptor signalling and TLR signalling pathways. These key pathways and their relate genes are listed in Table 5.1. Within these 7 pathways, TLR signalling pathway was with false discovery rate of 0.0114 and 9 genes were found to be involved in this pathway (Table 5.1).

In line with my results in Chapter 4, showing IL-37a and IL-37b inhibited TLR-induced inflammatory cytokine production in innate and adaptive immune cells, here both the IL-37 cytokines also suppressed several signalling components in the TLR pathway (Table 5.1 and Figure 5.4a). These included TLR3 that recognise viral double-stranded RNA via MyD88-independent pathway and, interestingly, irf5

involved in the MyD88-independent signalling for type I interferon production was also inhibited; the upstream signalling molecules TAB1 and RelA that enhance the IκB kinase (IKK) complex activity and nuclear translocation of the transcription factor NF-κB (Beutler *et al.,* 2006); Mapk11 and 12 that encode p38α mitogen-activated protein kinases (MAPK), NF-kB elements and Pik3cd (Table 5.1a); the Nos2 encodes nitric oxide synthase 2 which induces nitric oxide (NO) production. NO mediates bactericidal actions and is also involved in inflammation, enhances the synthesis of proinflammatory mediators such as IL-6 and IL-8 (Vlahopoulos *et al.,* 1999). This is consistent with my results in Chapter 4 showing that IL-37 suppresses Nos2 expression and NO production *in vitro* and *in vivo*. Furthermore, some molecules in T cell receptor (TCR) signalling pathway, including the cd3d and cd3g in TCR complex and TCR co-receptor genes cd4 and cd8b also inhibited. This suggests that both isoforms have profound regulatory effect on T cell-mediated response.

IL-37a and b suppressed genes were further analysed in triplicate and compared with WT control by heatmap (Figure 5.5a), to further confirm the gene downregulation and the repetitive of the assay.

The upregulated genes by both IL-37a and b were associated with 4 pathways (Figure 5.4b). These key pathways and their relate genes are listed in Table 5.2. 9 IL-37a and b inhibited genes and 6 enhanced genes were further analysed and compared by heatmap (Figure 5.5b).

Both isoforms also enhanced the expression of some signalling molecules expression in NF-kappa B pathway in T and B cell activation, including Lck and Zap70 involved in early T/B cell activation (Arulraj and Barik, 2018; Lo *et al.,* 2018) and the T cell growth cytokine/receptors il7 and il2rb, while the impact is still unknown. Some co-signalling molecules and molecules involved in apoptosis were also enhanced by both IL-37 isoforms, including icam1, fit3 and Bcl22b, Bcl2a, Gadd45b and Wt1, suggesting that both isoforms may be also associated with the regulation of co-stimulation and cell death. However, the biological meaning is still unclear.

Together with the result from Chapter 4, these results further reveal that IL-37a and b share similar functional ability in suppression of several signalling pathways and components induced by LPS in splenocytes.

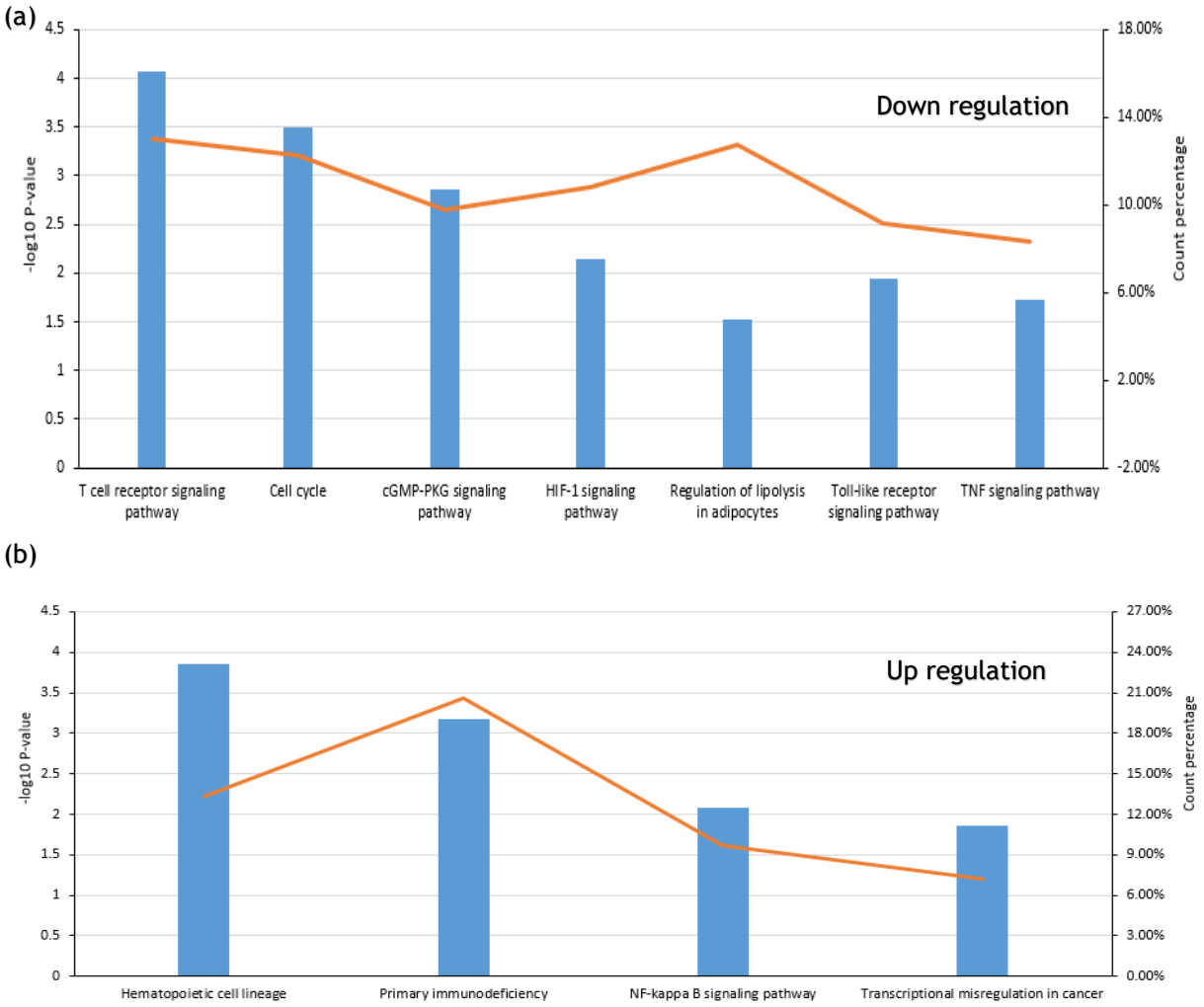



**Figure 5.4 The signalling pathways regulated by both IL-37a and b in LPS stimulated transgenic mice splenocytes**

The signalling pathways of the common genes regulated by both IL-37a and IL-37b in LPS-stimulated transgenic mice identified by the enrichment analysis on KEGG. The x-axis is the name of KEGG pathways; the left y-axis is associated p-value presented by (-log10^ p value) and the right y-axis is the count percentage of regulated genes in this analysis of the whole gene in each pathway; orange line is the count percentage value for each pathway. The cut-off value in this experiment is p value <0.05. a). Represents common pathways downregulated by IL-37a and b; b). The common pathways upregulated by IL-37a and b.

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| **T cell receptor signaling pathway** | 10 | 100 | 8.42E-05 | Cd3d,Cd3g,Cd4,Cd8b1,Csf2,Map2k7,Mapk11,Mapk12,Nfatc2,Rela |
| **Cell cycle** | 15 | 122 | 0.00032 | Bub1b,Ccna2,Ccnb1,Cdc25c,Cdc45,Cdk1,Cdkn2d,Chek2,Dbf4,Fzr1,Mcm6,Mcm7,Rb1,Tfdp2,Ttk |
| **cGMP-PKG signaling pathway** | 16 | 164 | 0.0014 | Adcy6,Adcy9,Adra2a,Atp2a3,Gna12,Gucy1a3,Gucy1b3,Insr,Mrvi1,Myl9,Mylk,Pde3a,Pde5a,Ppif,Prkg1,Trpc6 |
| **HIF-1 signaling pathway** | 11 | 102 | 0.0072 | Angpt1,Egln3,Eif4ebp1,Eno1,Insr,Mknk1,Mknk2,Nos2,Pfkl,Pik3cb,Prkca |
| **Regulation of lipolysis in adipocytes** | 7 | 55 | 0.0298 | Adcy6,Adcy9,Insr,Pik3cb,Prkg1,Ptger3,Ptgs1 |
| **Toll-like receptor signaling pathway** | 9 | 98 | 0.0114 | Cd80,Il12b,Irf5,Map2k7,Mapk11,Mapk12,Rela,Tab1,Tlr3 |
| **TNF signaling pathway** | 9 | 108 | 0.0188 | Ccl2,Creb3l4,Csf2,Icam1,Map2k7,Mapk11,Mapk12,Rela,Tab1 |

**Table 5.1 Common pathways and related genes downregulated by both IL-37a and b**

The key pathways selected from the Pathways analysis presented in Figure 5.4a; these pathways are presented here in detail. Background gene count is the total number of genes in this pathway; the name and number of the genes that are downregulated by IL-37a and b and involved pathway with false discovery rate (p value).

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| **Hematopoietic cell lineage** | 9 | 90 | 0.00014 | Flt3,H2-Aa,H2-Ab1,H2-DMb1,H2-DMb2,H2-Oa,Il7 |
| **Primary immunodeficiency** | 4 | 34 | 0.00066 | Cd79a,Lck,Tap1,Zap70 |
| **NF-kappa B signaling pathway** | 6 | 93 | 0.0084 | Bcl2a1c,Card11,Gadd45b,Lck,Ltb, Zap70 |
| **Transcriptional misregulation in cancer** | 11 | 167 | 0.0138 | Bcl11b,Bcl2a1c,Cdkn1a,Flt3,Gadd45b,Il2rb,Itgb7,Ngfr,Uty,Wt1 |

**Table 5.2. Common pathways and related genes upregulated by both IL-37a and b**

The key pathways selected from the Pathways analysis presented in Figure 5.4b, these pathways are presented here in detail. Background gene count is the total number of genes in this pathway; the name and number of the genes that are downregulated by IL-37a and b and involved pathway with false discovery rate (p value).

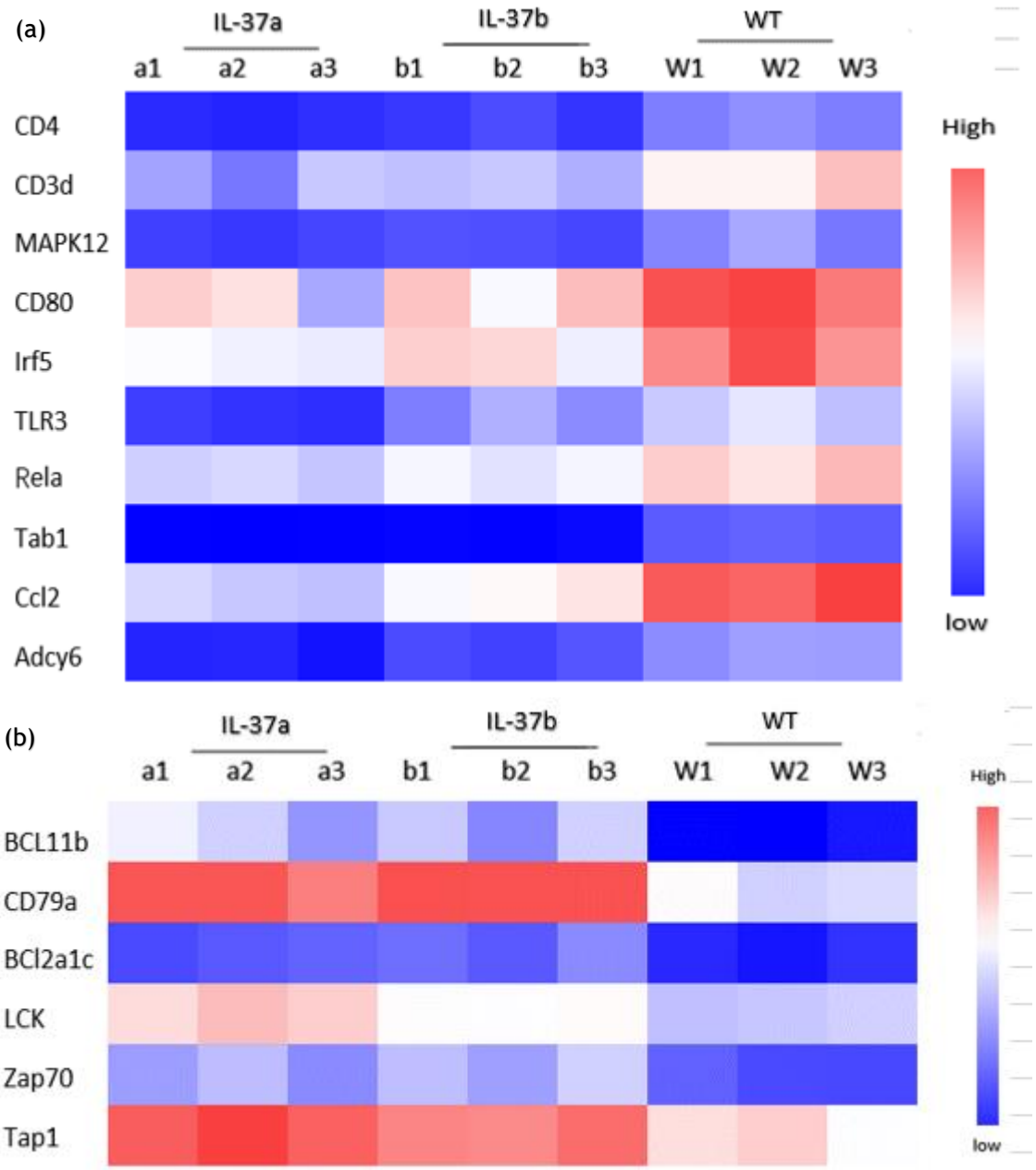



**Figure 5.5 Heat maps presentation of the common genes regulated by IL-37a and b compared to WT control**

The representative common regulated genes by IL-37a and b selected from tables 5.1 and 5.2 displayed in heat maps. The genes were analysed by microarray assay in triplicate. a) The representative genes that are downregulated by IL-37a and b; b) the representative genes that are upregulated by IL-37a and b compared with WT controls.

### 5.2.3 Identifying the signalling pathways/genes specifically regulated by IL-37a

The IL-37a specifically regulated genes identified in Figure 5.3 were further analysed for their association with signalling pathways as above. While IL-37a specifically inhibited 773 but enhanced 732 genes compared with IL-37b (Figures 5.3a and b), it downregulates more signalling pathways than it enhanced (Figures 5.6a and b). As shown in Figure 5.6a, IL-37a significantly downregulated genes in 5 signalling pathways and upregulated genes in 4 pathways. The downregulated pathways closely related to this study included TLR signalling, MAPK, IL-17 signalling pathways, salmonella infection and chemokine (Figure 5.6a and Table 5.3).

The results suggest that IL-37a may specifically suppress TLR response and signalling in several aspects: downregulated TLR4 and TLR8 expressions in MyD88-dependent TLR pathway together with IL-37a can also inhibit TLR3 expression (Figure 5.4a), these suggest that IL-37a can suppress both MyD88-dependent and independent TLR pathways; IL-37a also inhibited LPS-response by downregulate the expression of LPS-binding protein (LBP) which binds to the lipid A moiety of LPS, serves as an affinity enhancer for CD14, facilitating its association with LPS and signalling via TLR4 (Weiss and Barker, 2018); it also suppressed the expression of downstream TLR signalling elements Tab1 and 3 Map kinases, Mapk3, 13 and 14; IL-37a also specifically inhibited IL-17 signalling pathway, mainly by suppressing the Map kinases and the expression of pro-inflammatory cytokines/chemokines and receptors; S100a8 and S100a9 Binding to TLR4 and AGER activates the MAP-kinase and NF-kappa-B signalling pathways resulting in the amplification of the pro-inflammatory cascade; Igf1r-Receptor tyrosine kinase for the actions of insulin-like growth factor 1 (IGF1). The activated IGF1R is involved in cell growth and survival control (Elmadan *et al.*, 2019); Il12a (IL-12 a chain) is involved in Th1 polarisation and IFN-γ induction. 4 chemokine receptors Ccr1, 2, 3 and Cxcr2 are associated with the migration of a wide range of immune cells (Hölscher, 2004). IL-37a also affected drug metabolism pathway, however, its contribution to IL-37a-mediated regulatory effect in immunoregulation is currently unknown.

The genes/pathways specifically enhanced by IL-37a were also identified (Figure 5.6b, Table 5.4 and Figure 5.7 b). Two of the upregulated pathways by IL-37a were the cortisol synthesis and secretion and intestinal immune network for IgA production (Figure 5.6b and Table 5.4). IL-37a markedly increased expression of several genes in the two pathways, however, the biological function of the genes and pathways in IL-37a-mediated effect is currently unknown.

IL-37a specifically up and downregulated genes were further confirmed in triplicate and compared with WT control in heatmap (Figure 5.5a and b).

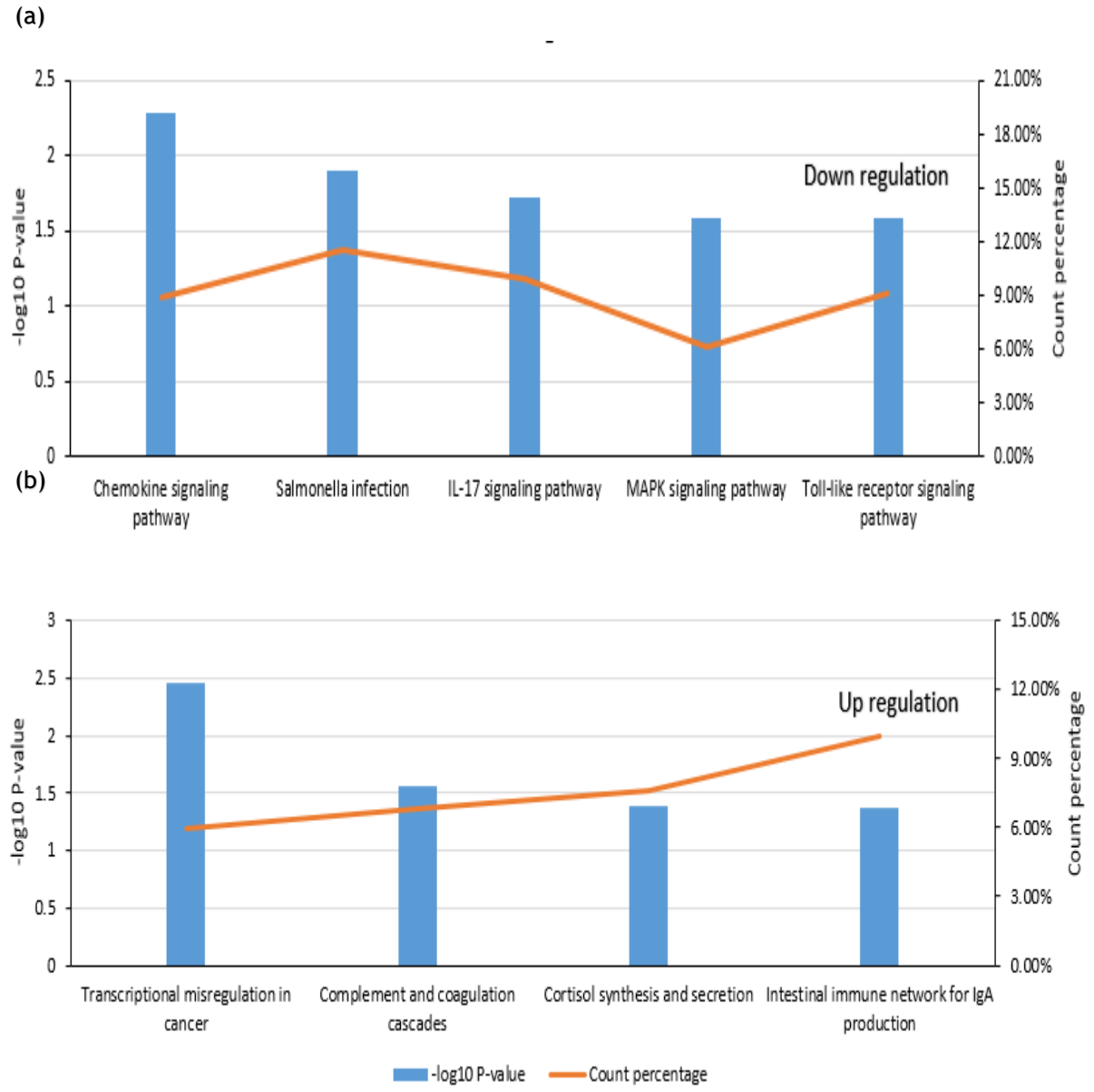



**Figure 5.6 The signalling pathways selectively regulated by IL-37a in LPS stimulated transgenic mice splenocytes**

The signalling pathways of the common genes regulated by IL-37a in LPS-stimulated transgenic splenocytes identified by the enrichment analysis on KEGG. The x-axis is the name of KEGG pathways; the left y-axis is associated p-value presented by (-log10^ p value) and the right y-axis is the count percentage of regulated genes in this analysis of the whole gene in each pathway; orange line is the count percentage value for each pathway. The cut-off value in this experiment is p-value <0.05. a) Represents pathways downregulated by IL-37a. b) are the pathways upregulated by IL-37a.

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| Chemokine signaling pathway | 16 | 179 | 0.0052 | Adcy5,Arrb1,Braf,Ccl9,Ccr1,Ccr2,Ccr3,Cxcl3,Cxcr2,Gnb5,Mapk3,Plcb1,Plcb2,Prkacb,Shc3,Tiam1 |
| Salmonella infection | 9 | 78 | 0.0125 | Cxcl3,Dync1li2,Flna,Lbp,Mapk13,Mapk14,Mapk3,Tlr4,Wasf2 |
| IL-17 signaling pathway | 9 | 91 | 0.019 | Cxcl3,Lcn2,Mapk13,Mapk14,Mapk3,Mmp9,S100a8,S100a9,Srsf1 |
| MAPK signaling pathway | 18 | 292 | 0.0259 | Arrb1,Braf,Cacnb2,Dusp6,Flna,Hgf,Igf1r,Mapk13,Mapk14,Mapk3,Pdgfd,Prkacb,Ptpn7,Rasgrp4,Rps6ka1,Rps6ka5,Tab1,Zak |
| Toll-like receptor signaling pathway | 9 | 98 | 0.0259 | Il12a,Lbp,Mapk13,Mapk14,Mapk3,Spp1,Tab1,Tlr4,Tlr8 |

**Table 5.3 KEGG pathways and related genes down regulated by IL-37a**

The key pathways selected from the Pathways analysis presented in Figure 5.6a these pathways are presented here in detail. Background gene count is the total number of genes in this pathway; the name and number of the genes that are downregulated by IL-37a and involved pathway with false discovery rate (p value).

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| Transcriptional misregulation in cancer | 10 | 167 | 0.0035 | Bcl2a1a,Bcl2a1b,Bcl2a1d,Cd86,Cdk9,Igfbp3,Myc,Plat,Rel,Tspan7 |
| Complement and coagulation cascades | 6 | 88 | 0.0276 | C1ra,C1s1,Plat,Procr,Serping1,Thbd |
| Cortisol synthesis and secretion | 5 | 66 | 0.0415 | Cacna1c,Ldlr,Nr5a1,Prkacb,Star |
| Intestinal immune network for IgA production | 4 | 40 | 0.0429 | Ccr10,Cd86,Cxcl12,Tnfrsf17 |

**Table 5.4 KEGG pathways and related genes up regulated by IL-37a**

The key pathways selected from the Pathways analysis presented in Figure 5.6b; these pathways are presented here in detail. Background gene count is the total number of genes in this pathway; the name and number of the genes that are downregulated by IL-37a and involved pathway with false discovery rate (p value).

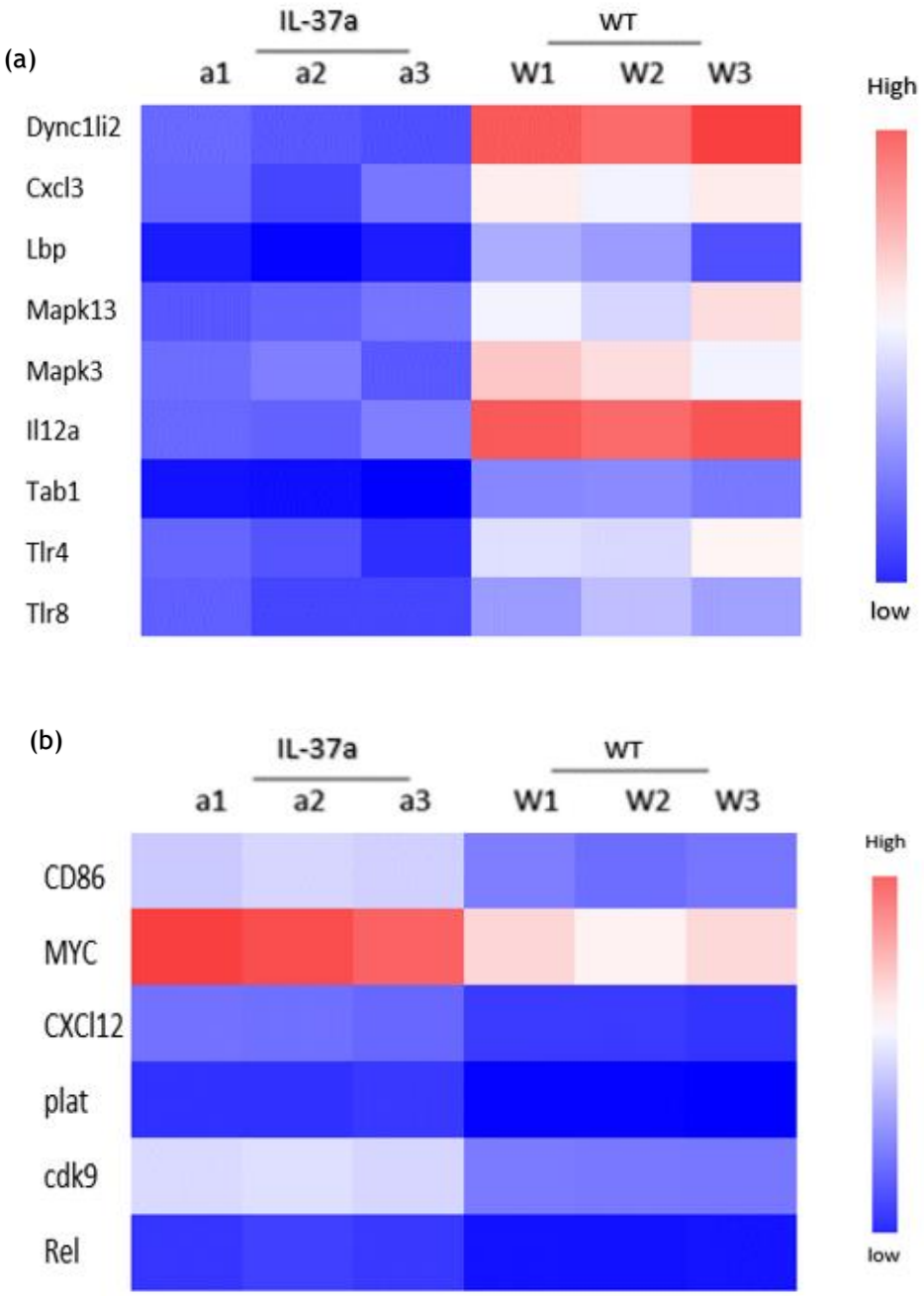



**Figure 5.7 Heat maps presentation of the genes regulated by IL-37a compared to WT control**

The representative common regulated genes by IL-37a selected from tables 5.3 and 5.4 displayed in heat maps. The genes were analysed by microarray assay in triplicates. a) the representative genes (table 5.3) that are downregulated by IL-37a; b) the representative genes (table 5.4) that are upregulated by IL-37a compared with WT controls.

### 5.2.4 Identifying the signalling pathways/genes specifically regulated by IL-37b

Using the same strategies, the IL-37b specifically regulated genes identified by RNA-seq analysis in Figure 5.3 were further analysed for their association with signalling pathways as above. As shown in Figure 5.8, IL-37b significantly downregulated genes in 4 KEGG pathways and enhanced in 3 pathways. The most significantly inhibited pathway was metabolic pathway (Figure 5.8a and Table 5.5).

The metabolism pathways include anabolism and catabolism pathways. Anabolism uses energy to construct the components of cells, for example proteins, while catabolism involves the breakdown of organic matter and stores energy as ATP. Host metabolism plays a critical role in the homeostasis and disease (Abbas *et al.*, 2011). The metabolic pathways can be influenced and regulated by many biological, physiological or chemical factors, including the immune mediators, temperature and the chemical environment, such as the pH level. Dysregulation of metabolism will lead to inflammation and disease (Zhao *et al.*, 2019).

As shown in Figures 5.8a, 5.9a and Table 5.5b, 85 genes in the metabolic pathways were significantly downregulated by IL-37b. We further analysed the 85 genes in the pathways and found that 83 of the genes are involved in the metabolic process and 77 involved in the cellular metabolic process (Figure 5.8c and Table 5.5b). This is different from IL-37a but is in consistent with the report that IL-37b may regulate cellular function by affecting cell metabolic pathways (Nold-Petry *et al.,* 2015).

The forkhead box O (FOXO) signalling pathway was the second key pathway regulated by IL-37b. FOXO family controls the expression of genes involved in cell apoptosis, cell-cycle, glucose metabolism and oxidative stress resistance. FOXOs can promote inflammatory response (Luo *et al.,* 2019). FOXO function can be activated by JNK and AMPK and inhibited by Akt/PKB), downstream of phosphatidylinositol 3-kinase (PI3K) (Léger *et al.,* 2006). However, the significance of regulation of FOXO pathway in IL-37b function is still less understood.

The most upregulated pathway was Fluid shear stress and atherosclerosis pathway (Fig, 5.8b). Shear stress caused by abnormal blood flow at the endothelial surface

of the blood vessel wall may trigger inflammatory response, the activation of pro-atherogenic pathways, NF-kB, AP1 and inflammatory cytokine/mediators and results in the development of atherosclerosis (Pan, 2017). This stress response is normally controlled by the host anti-atherogenic mechanisms, including upregulating the expressions of key anti-atherosclerosis factors, Kruppel-like factor 2 (KLF2), nuclear factor erythroid 2-like 2 (Nrf2) and eNOS in endothelial cell, that protective against atherosclerosis (Ota *et al.*, 2010). Consistent with the reports that IL-37b plays a protective role in the pathogenesis of atherosclerosis by anti-inflammation (Wang *et al.*, 2019), we also find that IL-37b could suppress AP1, NF-kB pathways and inflammatory cytokine production. Here IL-37b was able to enhance the expression of key anti-atherosclerosis factor KLF2. Thus, the protective effect of IL-37b in atherosclerosis could be via both the enhance anti-inflammatory and reduced pro-atherosclerosis mechanisms. However, the functions of other molecules enhanced by IL-37b in the pathway are unknown and need to be evaluated.

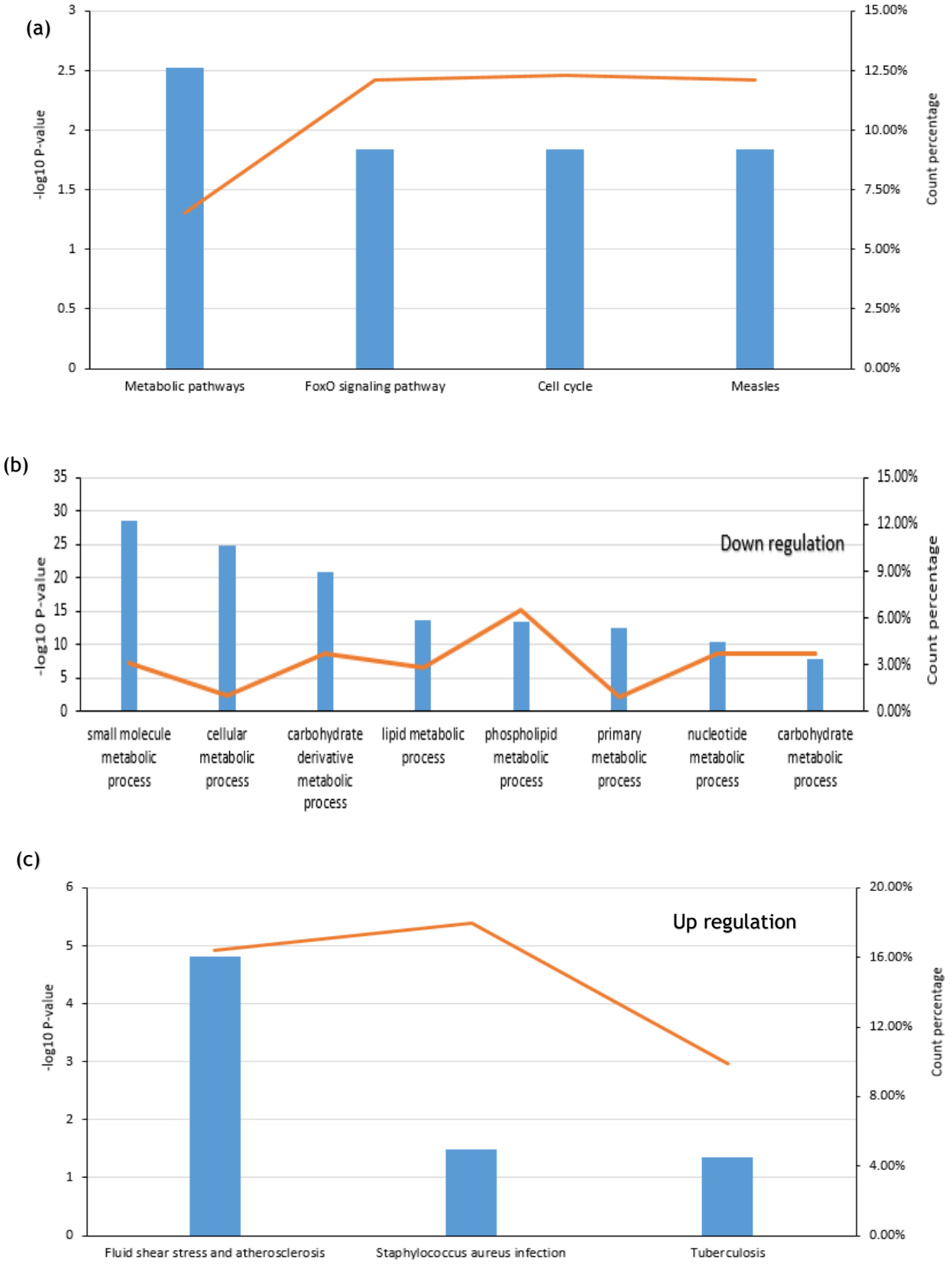



**Figure 5.8 The signalling pathways selectively regulated by IL-37b in LPS stimulated transgenic mice splenocytes**

The signalling pathways of the common genes regulated by IL-37b in LPS-stimulated transgenic mice splenocytes identified by the enrichment analysis on KEGG. The x-axis is the name of KEGG pathways; the left y-axis is associated p-value presented by (-log10^ p value) and the right y-axis is the count percentage of regulated genes in this analysis of the whole gene in each pathway; orange line is the count percentage value for each pathway. The cut-off value in this experiment is p-value <0.05.

a) represents pathways downregulated by IL-37b.

b) the detailed metabolic pathways downregulated by IL-37b

c) the pathways upregulated by IL-37b.

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| **Metabolic pathways** | 85 | 1296 | 0.003 | Acaa2,Acer2,Acmsd,Acox1,Acss1,Agpat3,Ak1,Alad,Aldh1a1,Aldh1a7,Aldh9a1,Atp5g1,Atp5g2,B3gat3,Blvrb,Btd,Cox7b,Cox8a,Dnmt3a,Dpm3,Extl3,Fahd1,Galk1,Galnt10,Gch1,Gclc,Gcnt1,Gm21987,Gpaa1,Hexa,Hk1,Hmgcl,Hmgcr,Hyal1,Hyal3,Idh3g,Itpkb,Lpin1,Mboat2,Mpi,Mpst,Mri1,Mthfr,Mtmr3,Nat2,Ndst3,Ndufa2,Ndufa6,Ndufb11,Ndufs7,Nt5c3,P4ha1,Pcyt1a,Pcyt1b,Pfkm,Pgp,Pi4k2b,Pigp,Pigq,Pigyl,Pla2g12a,Pla2g16,Plcb3,Plcg2,Pmm1,Pold1,Polr2c,Polr2d,Polr2e,Ppox,Prim2,Ptgs2,Pycr2,Rfk,Rrm2,Shmt2,Smpd1,St3gal1,St3gal3,Suox,Tusc3,Uqcr11,Uqcrq,Urod,Uros |
| **FoxO signaling pathway** | 16 | 132 | 0.0143 | Ccnb2,Cdkn1b,Fasl,Foxo4,Gabarapl1,Gabarapl2,Gadd45a,Irs2,Mapk12,Nlk,Plk1,Prkab1,Prkab2,Prkag1,Tgfb1,Tnfsf10 |
| **Cell cycle** | 15 | 122 | 0.0143 | Anapc11,Ccnb2,Ccne1,Ccne2,Cdc25b,Cdc6,Cdc7,Cdkn1b,Cdkn2c,Gadd45a,Plk1,Prkdc,Pttg1,Tgfb1,Ywhah |
| **Measles** | 16 | 132 | 0.0143 | Ccne1,Ccne2,Cd209b,Cdkn1b,Eif2ak1,Fasl,Gnb2l1,Hspa1a,Hspa1b,Il1b,Irf7,Mavs,Oas1a,Oas3,Prkcq,Tnfsf10 |

**Table 5.5a KEGG pathways and related genes down regulated by IL-37b**

The key pathways selected from the Pathways analysis presented in Figure 5.8a; these pathways are presented here in detail. Background gene count is the total number of genes in this pathway; the name and number of the genes that are downregulated by IL-37b and involved pathway with false discovery rate (p value).

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| **Small molecule metabolic process** | 47 | 1489 | 2.55E-29 | Acaa2,Acer2,Acmsd,Acox1,Acss1,Agpat3,Ak1,Aldh1a1,Aldh1a7,Atp5g1,Atp5g2,B3gat3,Btd,Cox8a,Galk1,Gch1,Gclc,Hk1,Hmgcl,Hmgcr,Hyal1,Hyal3,Idh3g,Lpin1,Mpi,Mpst,Mri1,Mthfr,Ndst3,Nt5c3,P4ha1,Pfkm,Pgp,Pla2g16,Plcb3,Plcg2,Pmm1,Ptgs2,Pycr2,Rfk,Rrm2,Shmt2,St3gal1,Suox,Uqcrq,Urod,Uros |
| **Cellular metabolic process** | 77 | 7348 | 1.84E-25 | Acaa2,Acer2,Acmsd,Acox1,Acss1,Agpat3,Ak1,Alad,Aldh1a1,Aldh1a7,Aldh9a1,Atp5g1,Atp5g2,B3gat3,Blvrb,Btd,Cox7b,Cox8a,Dnmt3a,Dpm3,Extl3,Galk1,Galnt10,Gch1,Gclc,Gcnt1,Gpaa1,Hexa,Hk1,Hmgcl,Hmgcr,Hyal1,Hyal3,Idh3g,Lpin1,Mboat2,Mpi,Mpst,Mri1,Mthfr,Mtmr3,Ndst3,Nt5c3,P4ha1,Pcyt1a,Pcyt1b,Pfkm,Pgp,Pi4k2b,Pigp,Pigq,Pigyl,Pla2g12a,Pla2g16,Plcb3,Plcg2,Pmm1,Pold1,Polr2c,Polr2d,Polr2e,Ppox,Prim2,Ptgs2,Pycr2,Rfk,Rrm2,Shmt2,Smpd1,St3gal1,St3gal3,Suox,Tusc3,Uqcr11,Uqcrq,Urod,Uros |
| **Carbohydrate derivative metabolic process** | 33 | 882 | 1.17E-21 | Acaa2,Acss1,Ak1,Atp5g1,Atp5g2,B3gat3,Cox8a,Dpm3,Extl3,Galk1,Galnt10,Gcnt1,Gpaa1,Hexa,Hk1,Hmgcl,Hmgcr,Hyal1,Hyal3,Mpi,Ndst3,Nt5c3,Pfkm,Pigp,Pigq,Pigyl,Pmm1,Rfk,Rrm2,St3gal1,St3gal3,Tusc3,Uqcrq |
| **Lipid metabolic process** | 27 | 961 | 1.74E-14 | Acaa2,Acer2,Acox1,Acss1,Agpat3,Aldh1a1,Dpm3,Gpaa1,Hexa,Hmgcl,Hmgcr,Lpin1,Mboat2,Mtmr3,Pcyt1a,Pcyt1b,Pgp,Pi4k2b,Pigp,Pigq,Pigyl,Pla2g12a,Pla2g16,Plcb3,Plcg2,Ptgs2,Smpd1 |
| **Phospholipid metabolic process** | 17 | 263 | 3.60E-14 | Agpat3,Dpm3,Gpaa1,Mboat2,Mtmr3,Pcyt1a,Pcyt1b,Pgp,Pi4k2b,Pigp,Pigq,Pigyl,Pla2g12a,Pla2g16,Plcb3,Plcg2,Smpd1 |
| **Primary metabolic process** | 64 | 7426 | 3.90E-13 | Acaa2,Acer2,Acox1,Acss1,Agpat3,Ak1,Aldh1a1,Atp5g1,Atp5g2,B3gat3,Cox8a,Dnmt3a,Dpm3,Extl3,Galk1,Galnt10,Gclc,Gcnt1,Gpaa1,Hexa,Hk1,Hmgcl,Hmgcr,Hyal1,Hyal3,Idh3g,Lpin1,Mboat2,Mpi,Mpst,Mri1,Mthfr,Mtmr3,Ndst3,Nt5c3,P4ha1,Pcyt1a,Pcyt1b,Pfkm,Pgp,Pi4k2b,Pigp,Pigq,Pigyl,Pla2g12a,Pla2g16,Plcb3,Plcg2,Pmm1,Pold1,Polr2c,Polr2d,Polr2e,Prim2,Ptgs2,Pycr2,Rfk,Rrm2,Shmt2,Smpd1,St3gal1,St3gal3,Tusc3,Uqcrq |
| **Nucleotide metabolic process** | 18 | 488 | 3.60E-11 | Acaa2,Acss1,Agpat3,Ak1,Atp5g1,Atp5g2,Cox8a,Galk1,Hk1,Hmgcl,Hmgcr,Idh3g,Mpi,Nt5c3,Pfkm,Rfk,Rrm2,Uqcrq |
| **Carbohydrate metabolic process** | 14 | 381 | 1.38E-08 | B3gat3,Galk1,Gclc,Hexa,Hk1,Hyal1,Hyal3,Mpi,Ndst3,Pfkm,Pgp,Pmm1,St3gal1,St3gal3 |

## Table 5.5b metabolic pathways downregulated by IL-37b

The key metabolic pathways selected from the pathway analysis presented in Figure 5.8b; these pathways are presented here in detail. Background gene count is the total number of genes in this pathway; the name and number of the genes that are downregulated by IL-37b and involved pathway with false discovery rate (p value).

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| Fluid shear stress and atherosclerosis | 23 | 140 | 1.50E-05 | Acvr1,Bcl2,Bmpr1a,Bmpr2,Ccl12,Cdh5,Dusp1,Edn1,Gpc1,Gstm1,Gstm4,Hmox1,Klf2,Mgst1,Mgst2,Mmp9,Ncf2,Sdc1,Sdc4,Tnfrsf1a,Txn1 |
| Staphylococcus aureus infection | 9 | 50 | 0.0326 | C2,C3ar1,Cfb,Cfh,Fcgr3,Fcgr4,Fpr1,Fpr2,Fpr3 |
| Tuberculosis | 15 | 172 | 0.0455 | Bcl2,Camk2d,Camp,Clec4e,Coro1a,Cycs,Eea1,Fcer1g,Fcgr3,Fcgr4,Hspa9,Ksr1,Ppp3ca,Tnfrsf1a,Traf6 |

**Table 5.6 KEGG pathways and related genes up regulated by IL-37b**

The key pathways selected from the Pathways analysis presented in Figure 5.8c; these pathways are presented here in detail. Background gene count is the total number of genes in this pathway; the name and number of the genes that are downregulated by IL-37b and involved pathway with false discovery rate (p value).

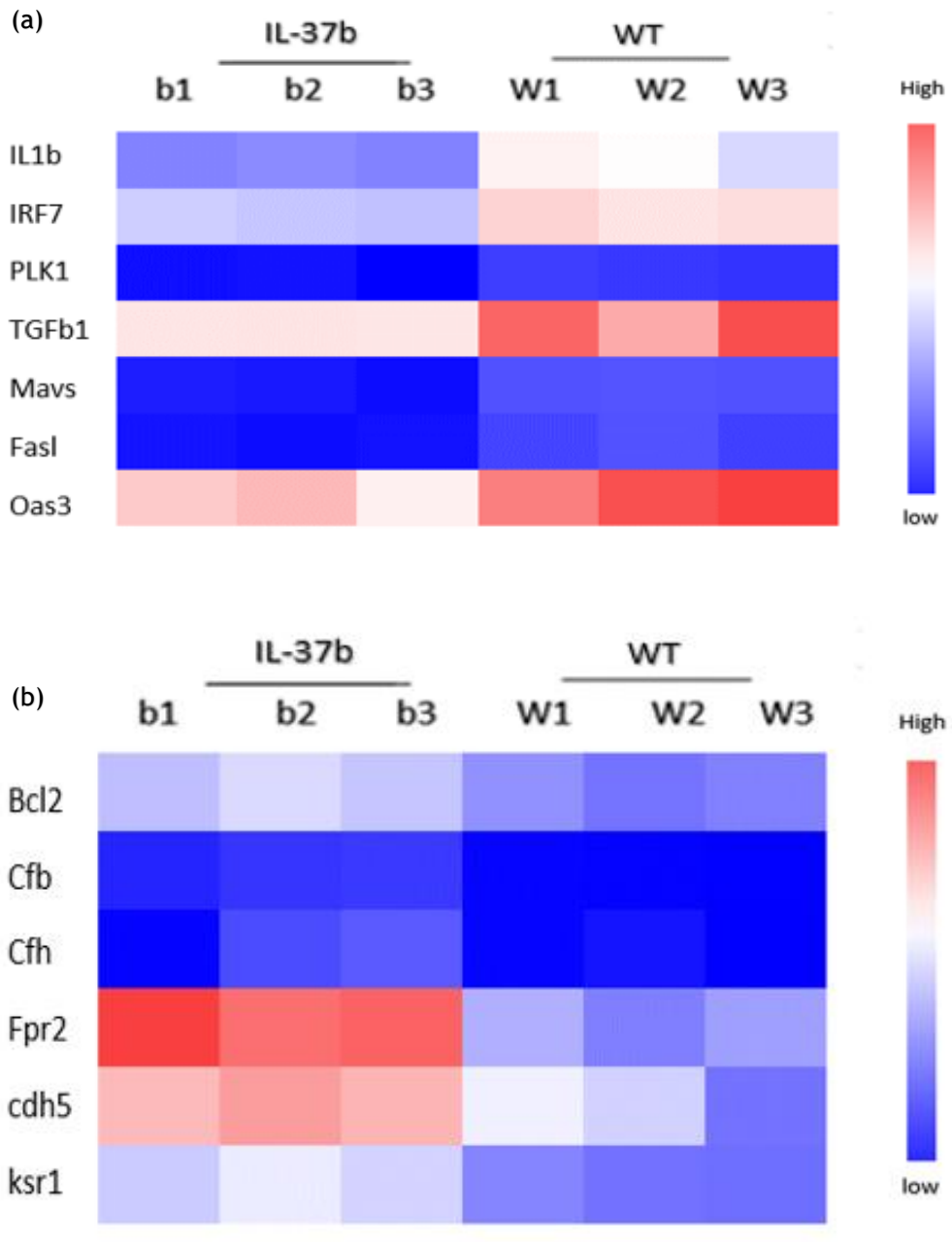



**Figure 5.9 Heat maps presentation of the genes regulated by IL-37b compared to WT control**

The representative common regulated genes by IL-37a selected from tables 5 and 6 displayed in heat maps. The genes were analysed by microarray assay in triplicate. a) The representative genes (table 5.5) that are downregulated by IL-37b; b) the representative genes (table 5.6) that are upregulated by IL-37b compared with WT controls.

### 5.2.5 Identifying the later LPS-induced genes differentially regulated by IL-37a and IL-37b in macrophage

Since many IL-37-related experimental results in Chapter 4 were generated from murine macrophage line RAW cells I sought next to further investigate the transcriptome of LPS-induced genes regulated by IL-37 isoforms using this cell line. Furthermore, considerable amount of my results in this context were obtained from macrophages about 18 h after TLR ligand stimulation, therefore, this transcriptome study was to focus on the later LPS stimulated gene profiles regulated by IL-37a and b in macrophages. RAW cells were transduced with plasmid expressing IL-37a, b or empty plasmid as a control, respectively. The cells were cultured in 24 wells plate and stimulated with 500ng/ml LPS for 18 hours. The total RNAs were isolated and transcriptome performed and analysed as before.

In order to identify the common and differential expressed genes between IL-37a and b, firstly, the differential expressed genes (DEG) between the WT and transgenic cells expression IL-37a or b, respectively, were identified using bio Venn programme and visualised by the area-proportional and overlapping Euler as before (data not shown). The DEG of IL-37a and b were used to further identify the common and specific genes regulated by the two isoforms using the same strategy. These results are shown in Figure 5.10.

Consistent with the transcriptome result from splenocytes (Figure 5.3), both cytokines were largely different in the regulation of LPS-induced genes, as shown in the specific regulated genes, further confirming that they vary in functions (Figure 5.10). 426 genes were downregulated by IL-37a and 1318 by IL-37b (Figure 5.10a). 249 genes were also upregulated by IL-37a and 1269 by IL-37b (Figure 5.10b). While both IL-37a and b dominantly inhibited LPS-induced gene expressions (Figures 5.10a and b). Furthermore, 371 genes were downregulated and 186 upregulated by both cytokines, further suggesting that they also share functional similarity in gene regulation.

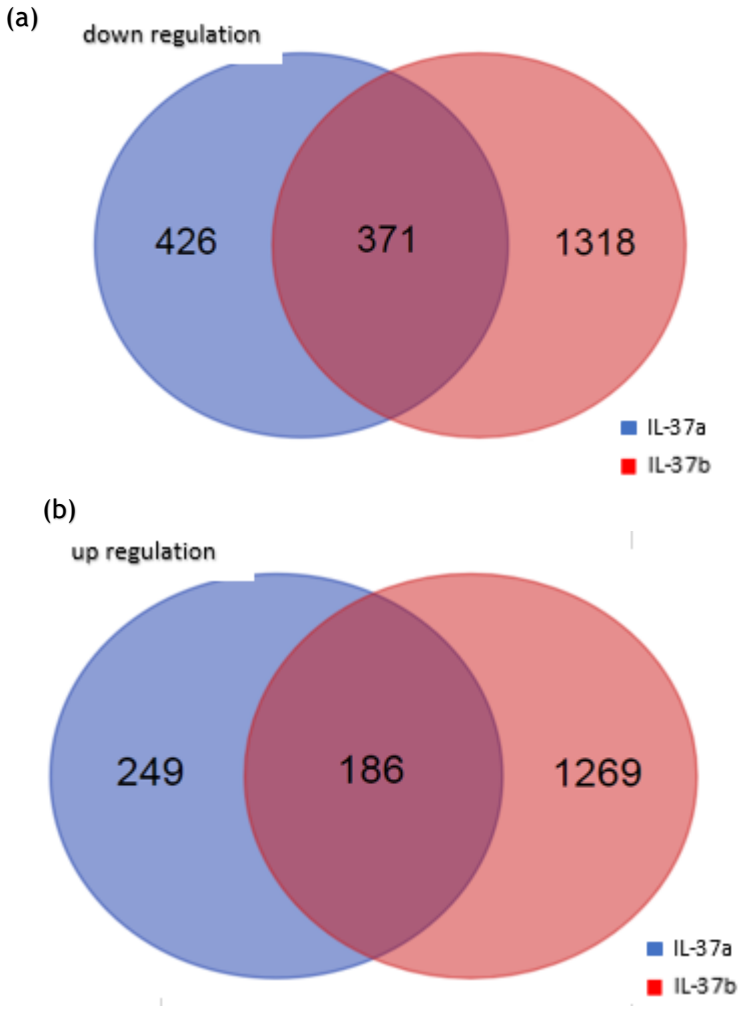



**Figure 5.10 Identification of common and specific genes differentially regulated by IL-37a and b in LPS stimulated transgenic RAW cells**

RAW cells with IL-37a and IL-37b transgene were compared with WT RAW cells after 500ng/ml LPS stimulation for 18 hours; total RNAs from each group were isolated for RNA-seq. The differential expressed genes identified using bio Venn programme and visualised by the area-proportional and overlapping (relationship) Euler. a) represents the genes that are downregulated by IL-37a and b; the red area shows genes downregulated by IL-37a only; the overlap area shows the genes that are downregulated by both IL-37a and b; while the blue area represents the genes that are downregulated by IL-37b only. b) shows the set of genes that are upregulated by IL-37a and b; specific genes upregulated by IL-37a shown in red area; the overlap area are common genes, which are upregulated by both IL-37a and b; the blue area are genes that are upregulated specifically by IL-37b.

### 5.2.6 Identifying the LPS-induced genes and signalling pathways commonly regulated by both IL-37a and b

The IL-37a and b regulated genes identified in Figure 5.10 were further analysed for their association with signalling pathways as above. Due to the time limitation, only the gene/pathways regulated by both IL-37a and b were analysed in detail here. The specifically gene/pathways regulated by either IL-37a or b in LPS-stimulated macrophages have not been but will be analysed soon.

Consistently, both cytokines downregulated more signalling pathways than they enhanced (Figure 5.11a and b). As shown in Figure 5.11a, IL-37a and b significantly downregulated genes in 10 signalling pathways and only upregulated genes in 5 pathways in macrophages. The top two significantly inhibited pathways were the TNF and chemokine signalling pathways (Figure 5.11a and Table 5.7). This is consistent with the regulatory role of IL-37 in cytokine/chemokine expression and function in diseases (Nold-Petry *et al.*, 2015; Petry *et al.*, 2015; Nold *et al.*, 2010).

Also, in agreement with our results in Chapter 4, IL-37a and b inhibited 17 genes in TNF pathway, including Tnfrsf1b (encoding TNF-α receptor 1), signalling molecules Fos, Mapk13 and ptgs2 and several chemokines/cytokines (Figure 5.11a and Table 5.7). In line with this, both isoforms significantly suppress the chemokine signalling pathway and downregulation of 16 chemokines/receptors, many of the chemokine/receptors are involved in inflammatory response (Inohara and Nuñez, 2001).

A number of inflammatory cytokine/receptor genes, including Il1b, Il4r, Il6, Il7r, Il18 were significantly inhibited by both isoforms (Table 5.7). Furthermore, IL-37a impaired the expression of key genes in Jak-STAT signal pathway, including stat3 which is important in cytokine induction. IL-10, TGF-β, Socs1 and 3 are important cytokine regulators, but also suppressed by IL-37 a and b, suggesting that IL-37 suppressing cytokines may not via these classic immunoregulators.

Also agreeing with my previous results in Chapter 4, showing IL-37a and IL-37b inhibited TLR response in macrophages, here both the cytokines also suppressed several signalling components in the TLR pathway (Table 5.7 and Figures 5.11a, 12a). These included the most important TLR adaptor MyD88 that plays initial role in TLR signalling (Medzhitov, 2001), the downstream signalling molecules Mapk13 that encode p38α mitogen-activated protein kinases (MAPK) and Irf7 (Table 5.7 and Figure 12a).

The expression of 18 genes in NOD-like receptor (NLR) signalling pathway were also inhibited by IL-37b in the LPS-stimulated cells. NLR is required for the inflammasome formation and IL-1ß and IL-18 maturation and secretion (Kavathas et al., 2013; Moreira and Zamboni, 2012). its impact on LPS/TLR4 signalling need to be evaluated.

Both the isoforms also significantly upregulated genes in 5 pathways (Figures 5.11b and 5.12). The top enhanced pathway was the biosynthesis of amino acids. However, the biological and immunological significance of these remain unknown.

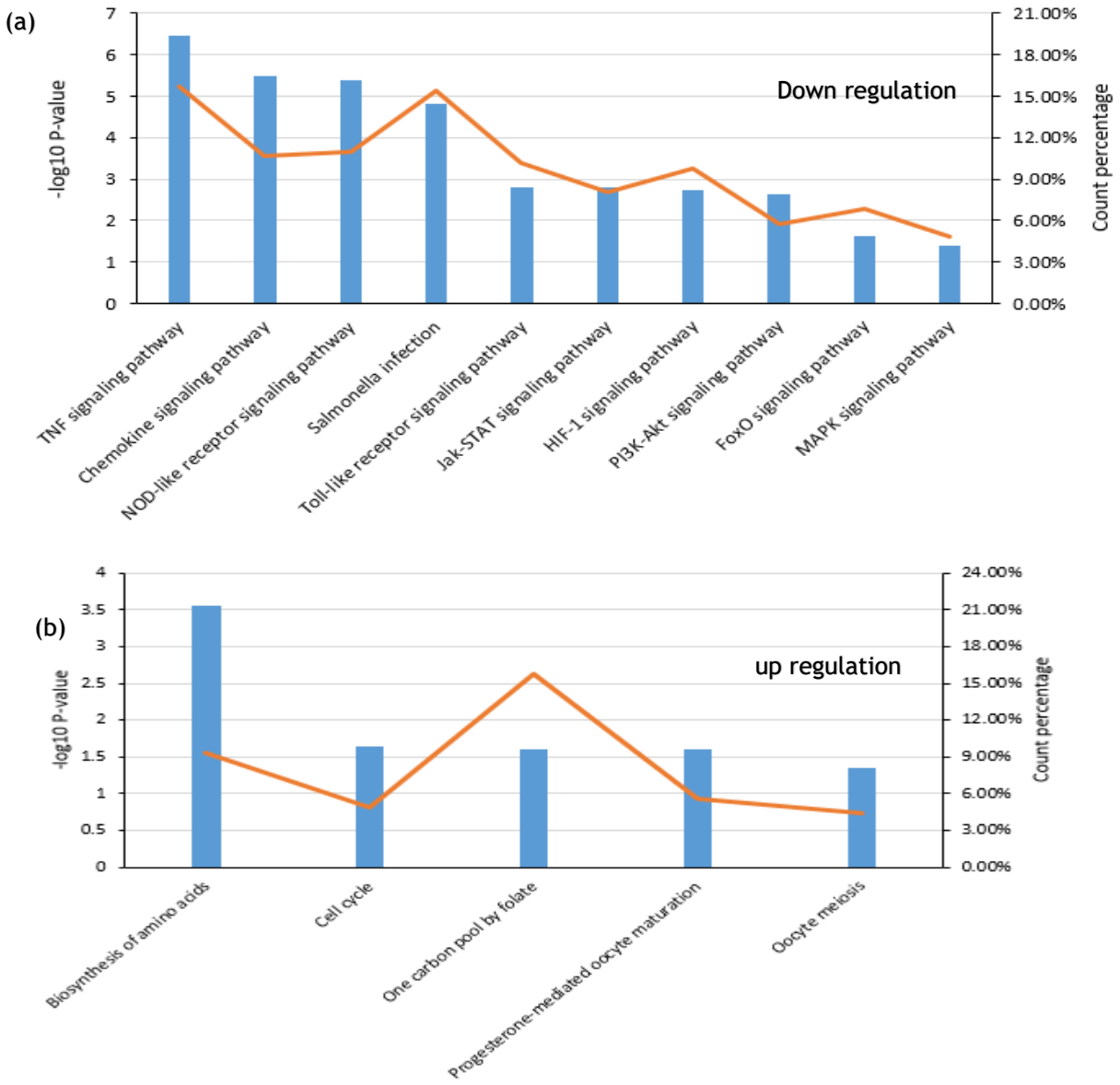



**Figure 5.11 The signalling pathways regulated by both IL-37a and b in LPS stimulated transgenic RAW cells**

The signalling pathways of the common genes regulated by both IL-37a and IL-37b in LPS-stimulated RAW cells identified by the enrichment analysis on KEGG. The x-axis is the name of KEGG pathways; the left y-axis is associated p-value presented by (-log10^ p value) and the right y-axis is the count percentage of regulated genes in this analysis of the whole gene in each pathway; orange line is the count percentage value for each pathway. The cut-off value in this experiment is p-value <0.05. a) Represents common pathways downregulated by IL-37a and b. b) are the common pathways upregulated by IL-37a and b.

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| **TNF signaling pathway** | 17 | 108 | 3.45E-07 | Bcl3,Ccl12,Ccl2,Ccl5,Creb5,Csf1,Cxcl10,Cxcl2,Cxcl3,Fos,Il1b,Il6,Lif,Mapk13,Ptgs2,Socs3,Tnfrsf1b |
| **Chemokine signaling pathway** | 19 | 179 | 3.38E-06 | Ccl12,Ccl2,Ccl22,Ccl3,Ccl4,Ccl5,Ccl6,Ccl7,Ccl9,Ccr1,Ccr7,Cxcl10,Cxcl2,Cxcl3,Cxcr3,Cxcr5,Gng7,Src,Stat3 |
| **NOD-like receptor signaling pathway** | 18 | 164 | 4.11E-06 | AI607873,Antxr2,Ccl12,Ccl2,Ccl5,Cxcl2,Cxcl3,Gbp3,Gbp7,Il18,Il1b,Il6,Irf7,Mapk13,Nlrc4,Oas2,Oas3,Trpm2 |
| **Salmonella infection** | 13 | 78 | 1.56E-05 | Ccl3,Ccl4,Cxcl2,Cxcl3,Fos,Il18,Il1a,Il1b,Il6,Mapk13,Nlrc4,iNOs,MyD88 |
| **Toll-like receptor signaling pathway** | 12 | 98 | 0.0016 | Ccl3,Ccl4,Ccl5,Cd80,Cxcl10,Fos,Il1b,Il6,Irf7,Mapk13,MyD88,Tbk1 |
| **Jak-STAT signaling pathway** | 13 | 161 | 0.0016 | Aox2,Cdkn1a,Csf3,Il10,Il11,Il4ra,Il6,Il7r,Lif,Osm,Socs1,Socs3,Stat3 |
| **HIF-1 signaling pathway** | 10 | 102 | 0.0019 | Camk2a,Cdkn1a,Eno2,Eno3,Hmox1,Il6,iNOs,Pfkfb3,Serpine1,Stat3 |
| **PI3K-Akt signaling pathway** | 20 | 349 | 0.0023 | Cdkn1a,Creb5,Csf1,Csf3,Epha2,Gng7,Il4ra,Il6,Il7r,Itgav,Itgb4,Itgb7,Lpar1,Mdm2,Nr4a1,Osm,Pik3ap1,Syk,Thbs1,Thbs3 |
| **FoxO signaling pathway** | 9 | 132 | 0.0235 | Cdkn1a,Homer3,Il10,Il6,Il7r,Mapk13,Mdm2,Stat3,Tgfb3 |
| **MAPK signaling pathway** | 14 | 292 | 0.0423 | Csf1,Dusp1,Dusp2,Dusp5,Epha2,Fos,Hspa1b,Il1a,Il1b,Mapk13,Nr4a1,Ptpn5,Tgfb3,Zak |

**Table 5.7 Common pathways and related genes downregulated by both IL-37a and b**

The key pathways selected from the Pathways analysis presented in Figure 5.11a; these pathways are presented here in detail. Background gene count is the total number of genes in this pathway; the name and number of the genes that are downregulated by IL-37a and b and involved pathway with false discovery rate (p value).

| Term description | Observed gene count | Background gene count | False discovery rate | Matching proteins in your network (labels) |
|---|---|---|---|---|
| Biosynthesis of amino acids | 7 | 75 | 0.00072 | Aldh18a1,Ass1,Cth,Gpt2,Psph,Pycr1,Shmt2 |
| One carbon pool by folate | 3 | 19 | 0.0482 | Aldh1l2,Mthfd2,Shmt2 |
| Cell cycle | 6 | 122 | 0.0482 | Bub1b,Ccna2,Cdc20,Cdc25c,Cdkn2c,Plk1 |
| Progesterone-mediated oocyte maturation | 5 | 90 | 0.0482 | Ccna2,Cdc25c,Igf1,Kif22,Plk1 |

**Table 5.8 Common pathways and related genes upregulated by both IL-37a and b**

The key pathways selected from the Pathways analysis presented in Figure 5.11b; these pathways are presented here in detail. Background gene count is the total number of gene in this pathway; the name and number of the genes that are upregulated by IL-37a and b and involved pathway with false discovery rate (p value).

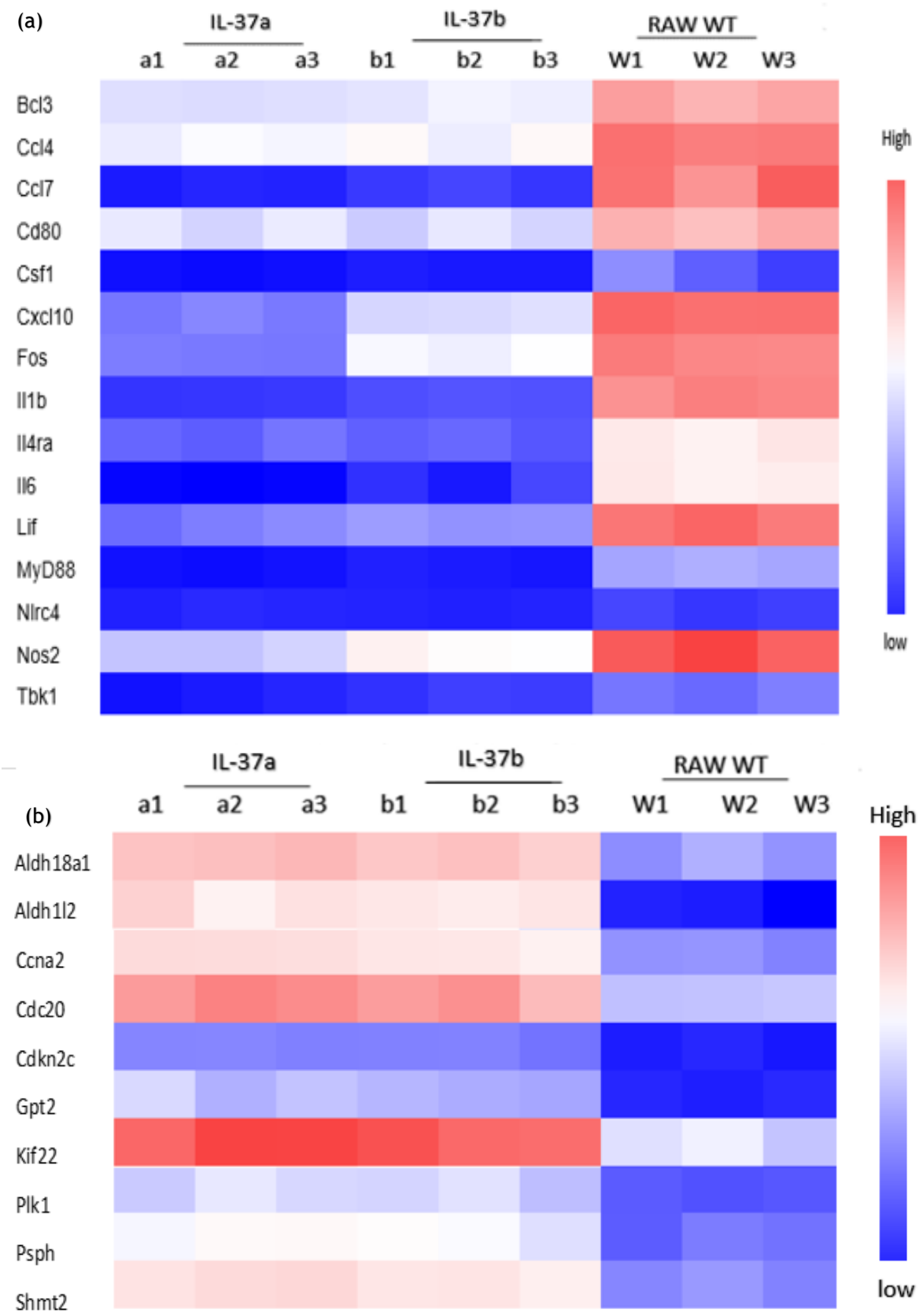
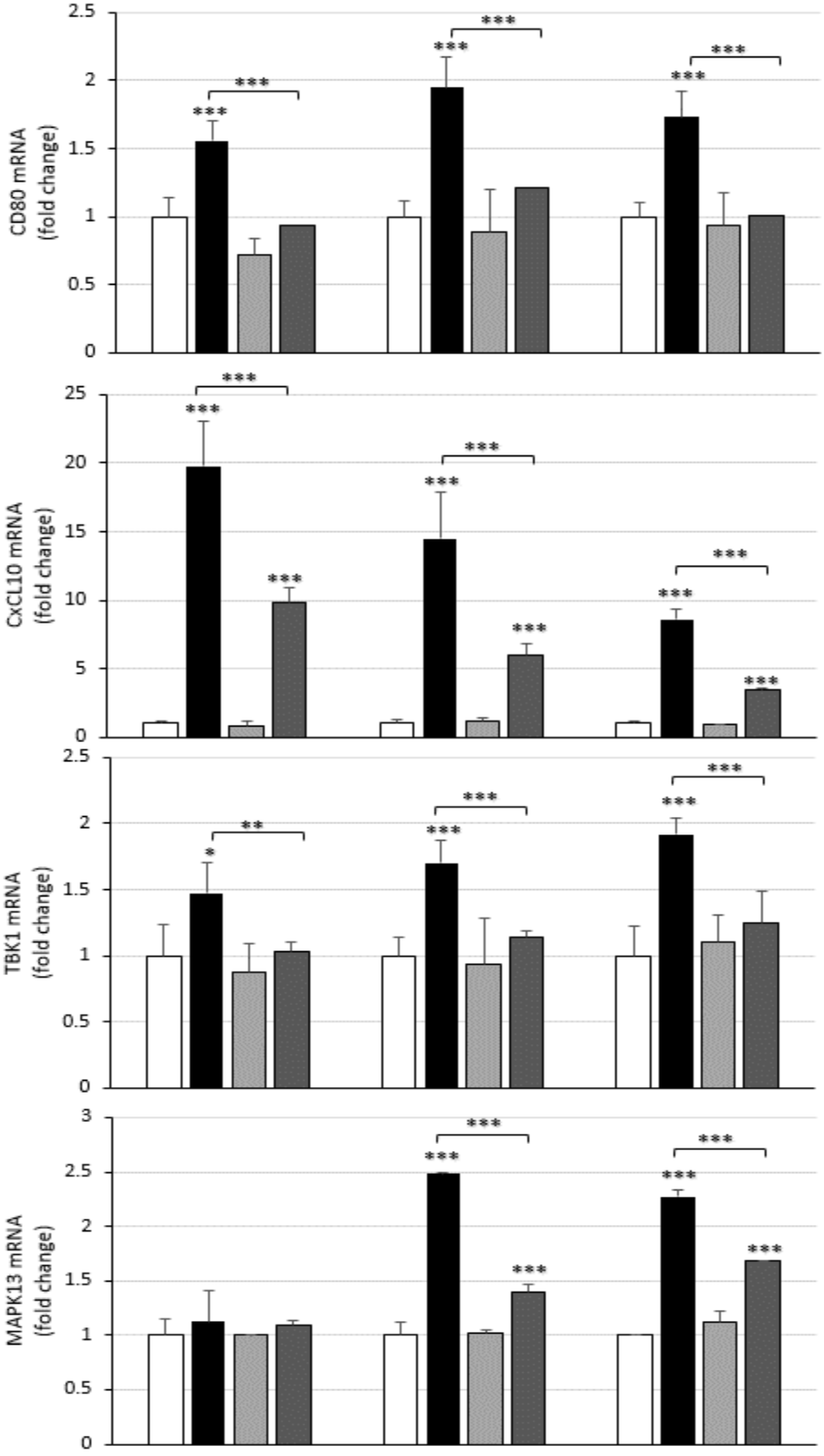



**Figure 5.12 Heat maps presentation of the common genes regulated by IL-37a and b compared to WT control**

The representative common regulated genes by IL-37a and b selected from tables 5.1 and 5.2 displayed in heat maps. The genes were analysed by microarray assay in triplicate. a) The representative genes (table 5.1) that are downregulated by IL-37a and b; b) the representative genes (table 5.2) that are upregulated by IL-37a and b compared with WT controls.

### 5.2.7 Validation of the differential expression genes induced by LPS and regulated by IL-37a in splenocytes *in vitro*

Some of the differentially expressed genes identified by RNA-seq between the WT and IL-37a groups were further validated in LPS-stimulated primary splenocytes from IL-37aTg mice by QPCR (Figure 5.13). The genes were randomly selected. We confirmed that, the same as the results in RNA-seq, IL-37a also downregulated CD80, Cxcl10, TBK1, MAPK13, STAT3 and Fos in time-dependent manner. There are 3 of the upregulated genes by IL-37a in RNA-seq also confirmed including Aldh18a1, psph and Cth (Figure 5.14). These results suggested that the RNA-seq results are reliable.




| Time(hours) | 5 | | | | 12 | | | | 18 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | + | + | — | — | + | + | — | — | + | + | — | — |
| IL-37aTg | — | — | + | + | — | — | + | + | — | — | + | + |
| LPS | — | + | — | + | — | + | — | + | — | + | — | + |

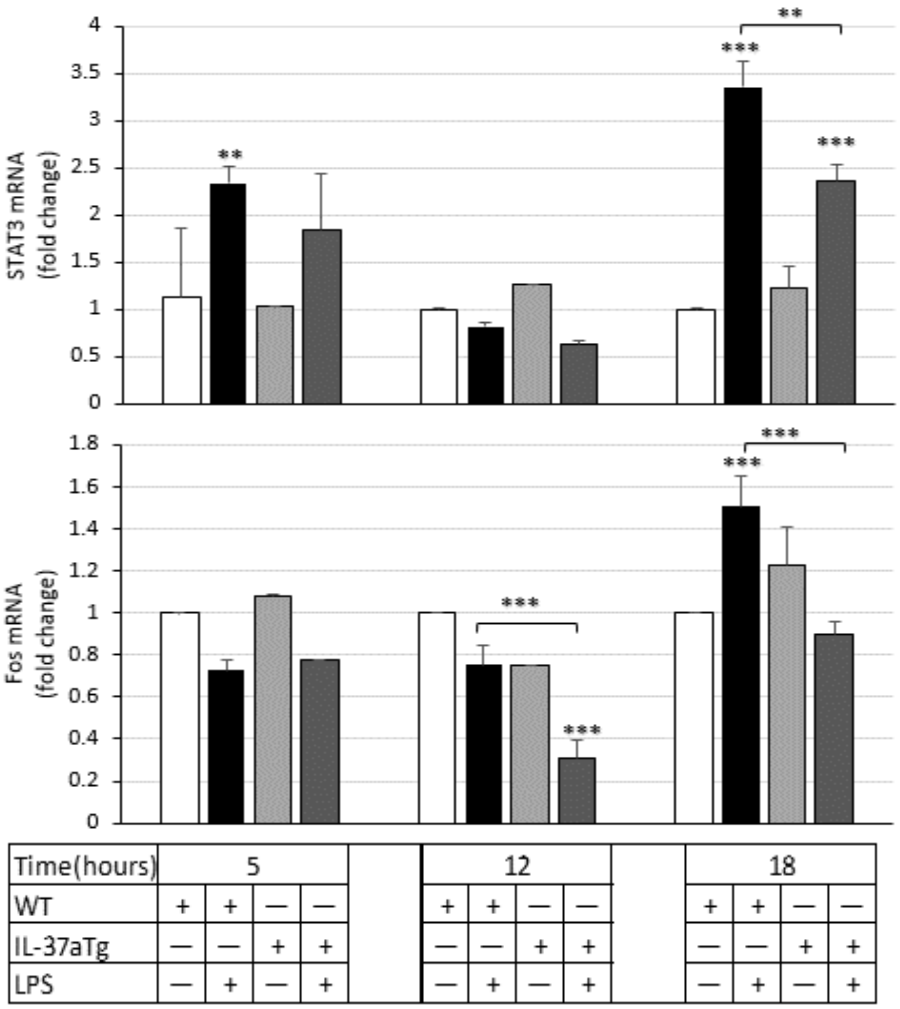



**Figure 5.13 IL-37a up regulated gene validation in murine splenocytes *in vitro***

To validate the gene experiment and confirm the time dependent trend of IL-37a down regulated genes. Splenocytes from WT and IL-37aTg (1x10^6/ml) were cultured in the presence or absence of 500ng/ml of LPS for 5, 12 and 18 hours. Total RNAs were extracted from total cell lysate and the expression levels of interest genes from IL-37a downregulation group (CD80, Cxcl10, TBK1, MAPK13, STAT3 and Fos) were measured by qPCR. Using ANOVA, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

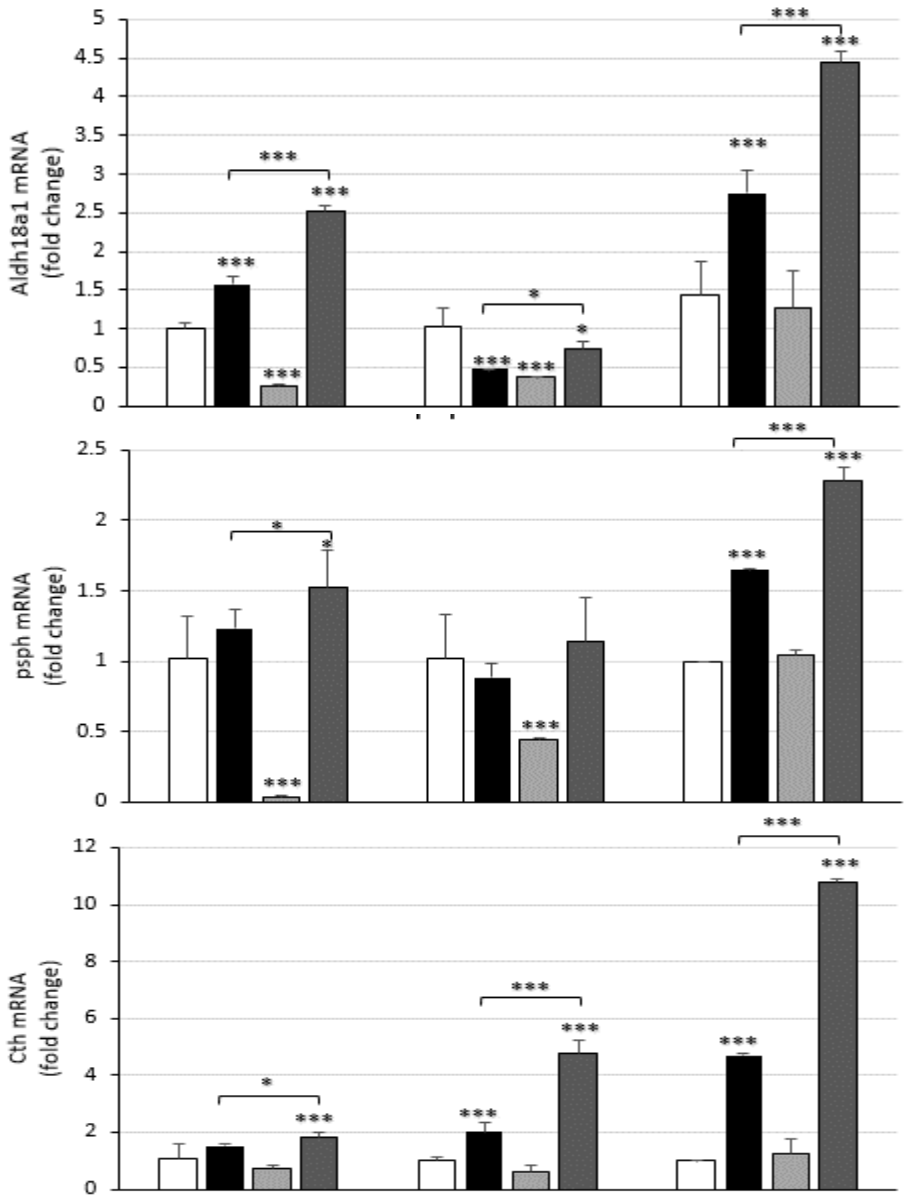



**Figure 5.14 IL-37a upregulated gene validation in murine splenocytes *in vitro***

To validate the gene experiment and confirm the time dependent trend of IL-37a upregulated genes. Splenocytes from WT and IL-37aTg (1x10^6/ml) were cultured in the presence or absence of 500ng/ml of LPS for 5, 12 and 18 hours. Total RNAs were extracted from total cell lysate and the expression levels of interest genes from IL-37a upregulation group (Aldh18a1, psph and Cth) were measured by qPCR. Using ANOVA, data are means ± SD, and are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

## 5.3 Discussion

In summary, the transcriptomic results demonstrated for the first time:

1. Compared to the WT control, IL-37a significantly downregulated 2151 genes and upregulated 1068 genes in the LPS-stimulated cells, indicating that IL-37a has a broad regulatory effect on global gene expression, not only suppressing but enhancing gene expressions.

2. Compared to the IL-37b, IL-37a specifically inhibited 773 and also enhanced 732 genes, suggesting that IL-37a differs from IL-37b in gene regulation.

3. Compared with IL-37b, the common regulated genes between the two IL-37 isoforms were also identified, explaining the functional similarity between the two isoforms.

4. the common and unique signalling pathways between the two isoforms were also revealed using innate DB gene bank and KEGG enrichment analysis. These have provided molecular explanations, at least in part, for the IL-37-mediated regulatory effect in the previous experimental observations in Chapter 4.

The RNA-seq experiment has been established in our Chinese collaborator's lab (by R.Wei). The spleen cells are harvested from WT C57BL6 mice and C57BL6 mice with IL-37a and IL-37b transgene (CMV promotor). We extracted RNA from these splenocytes and generated transcriptomic analysis. The IL-37a and IL-37b gene expression from these mice are similar in mRNA level and protein level. The protein level is around 15ng/ml in serum and the mRNA level of IL-37 is stable in these two lines. Therefore, the gene expression can be controlled and to maintain in similar level, their RNA-seq result is comparable.

We use primary cell instead of RAW macrophage cell line here for RNA-sequencing. One of the most important reason for that is RAW cells are transformed and are not functional for certain signalling pathways like Ah receptor signalling. Moreover, the

transformed macrophage cell lines are more sensitive to the stimulation compare with primary cells, which may not reflect the real condition and gene expression. Therefore, except RAW cells, we also use splenocytes from WT and IL-37a/b transgenic mice to seek for the regulation mechanism of IL-37a and b in TLR stimulation.

The transcriptomic analysis further demonstrated that IL-37a is a previously unrecognised immunoregulatory cytokine and play critical role in the regulation of TLR-signalling and beyond. In addition, IL-37a differs from IL-37b in gene regulation and signalling and may be a potential therapeutic intervention for inflammatory diseases.

Consistent with our results that show IL-37a and IL-37b share functional similarity in the regulation of immune cell function (Chapter 4), considerable number of genes in LPS-stimulated cells were regulated by both IL-37a and IL-37b in the transcriptomics analysis. While the detailed mechanism still need to be elucidated, because the two isoforms share the IL-1-like cytokine domain for receptor binding and signal via the same receptor, it is mostly likely that the common genes are regulated via the common IL-37 receptor-dependent pathway.

Intriguingly, apart from the common regulated genes/pathways, many of the genes/pathways were specifically regulated by either IL-37a or IL-37b, respectively. For instance, the most downregulated genes/pathways by IL-37b were the metabolic pathways, while IL-37a mainly affects the immune response, including chemokine and TLR response. The result further demonstrates that IL-37a and IL-37b differ from each other, not only in biology but also in gene regulation and signalling. However, at the moment, it is poorly understood how the two IL-37 isoforms regulate gene profile differently. I believe that this is largely due to their difference in N-terminal sequence of the two IL-37 isoforms, which may cause their difference in nuclear translocation and bioactivity.

It has been shown that matured, but not the full-length IL-37b is able to translocate into cell nucleus via smad3 (Luo *et al.,* 2017). Using the IL-37b transgenic mice lacking the IL-1R8, it was further demonstrated that nuclear located mature IL-37b

can regulate some LPS-induced cytokines in an IL-1R8-independent manner *in vitro* and *in vivo* (Nold *et al.*, 2010; Moretti *et al.*, 2014). However, the subsequent report from the same research group showed that receptor-independent regulatory pathway of IL-37b was not important *in vivo* in LPS-induced shock (Nold-Petry *et al.*, 2015). Therefore, the regulatory effect of the mature IL-37b in cell nucleus may not be important, at least in LPS shock.

Because the unique N-terminal of the full-lengthy IL-37a (FL-IL-37a) contains a putative NLS (Nold-Petry *et al.*, 2015), we investigated the ability of the NLS in IL-37a translocation. Our preliminary results indicated that indeed, the majority FLIL-37a proteins were translocated into cell nucleus via their NLS (R. Wei, unpublished result). This is different from FLIL-37b. Thus, we speculate that the genes specifically regulated by IL-37a may be predominantly attributed to the nuclear FLIL-37a in a receptor-independent manner. Further experiments are needed to define the hypothesis by using the mice strain that expresses FLIL-37a but not the IL-1R8. While the molecular targets and mechanism of action of NIL-37a in the nucleus are still unknown and currently under investigation in our lab using Chip-sequence, it is known that the NLS containing N-terminal of IL-1α is sufficient to regulate cell function by translocation to the nucleus and binding to transcription activators and chromatin (Werman *et al.*, 2004b).

Since the putative elastase-matured IL-37a protein (R22-D192) shares 98% homology with the caspase-1 matured IL-37b (V46-D218), it is possible that after processing, matured IL-37a may also be translocated to the cell nucleus through the same Smad3-mediated mechanism as mature IL-37b. However, our preliminary result suggests that, different from the FLIL-37a, majority of mature IL-37a proteins are located in the cell plasma, not cell nucleus (R.Wei, unpublished result). The reason is still unknown, but it is likely that FL but not mature IL-37a is primarily responsible for the gene regulation in the cell nucleus.

Nevertheless, our results suggest that FLIL-37a is a dual functional regulatory molecule which regulates genes and cell function by two mechanisms: different from FlIL-37b; FLIL-37a can serve as a nuclear regulatory factor when translocated into

the cell nucleus; similar to IL-37b, FL and mature IL-37a can also act as a cytokine when released from producing cells and trigger signalling via IL-37 receptor.

Since many of my results in Chapter 4 studied the regulatory effect of IL-37a and b on LPS response I therefore studied their role in the regulation of TLR4 signalling here in more detail. Our transcriptomic analysis revealed that IL-37a and IL-37b control LPS/TLR4 signalling transduction by targeting multiple signalling cascades in the pathway and via common and specific mechanisms (Figure 5.15).

Both isoforms inhibited the expression of TLR4 co-receptor CD14 and the key adaptor protein MyD88. All these will prevent the initiation of LPS/TLR4 signalling. Both isoforms can further suppress the downstream TLR signalling, from the upstream signalling molecules in MyD88-dependent pathways including IRAK, TAB, MAPK, NF-kB and JAK-STAT pathways to the downstream JAK-STAT and proinflammatory cytokine and mediators productions.

Apart from the shared capacity in gene regulation, both isoforms can also act independently in gene modulation and control TLR signalling by different mechanisms. Furthermore, IL-37a appeared to be more effecting on the regulation of the MyD88-dependent pathway, and IL-37b on the TRIF-dependent pathway (Figure 5.15). As such, IL-37a, but not IL-37b, specifically inhibited the expression of TLR4 and also the LPS-binding protein (BLP), and this will affect LPS binding to TLR4 and signalling. Thus, IL-37a may prevent the initiation of TLR4 signalling from the very early stage. IL-37a also inhibited IRF5 and IKK expression.

In the regulation of TRIF-dependent pathways (Figure 5.15), IL-37b might play more role than IL-37a in this. IL-37b specifically suppressed TRIF, the key adaptor molecule in the pathway. On the other hand, IL-37b also inhibited the key signalling molecules Irf3 and AKT. Both isoforms further inhibited TBK1, together; this may lead to the downregulation of type I IFN production.

Altogether, these IL-37a and b-mediated effects will impair the LPS-induced production of inflammatory cytokines, chemokines and mediators and also ameliorate inflamed leucocyte migration, and immune reactions, and thereafter

decrease inflammatory response. Hence, the selective induction of IL-37, in particular IL-37a, may represent a novel strategy for improving immunotolerance against dysregulated inflammatory response. In addition, this analysis also provides a possible molecular explanation for the IL-37a-mediated regulatory effect on salmonella infection and NO production in Chapter 4.

At least 3 surface molecules of salmonella can be recognised by different TLRs, the LPS, lipoproteins and flagella (Zhan *et al.,* 2015). LPS/TLR4 and flagellin/TLR5 signals and perhaps the most important in salmonella-mediated immune response and pathogenesis (Li *et al.,* 2012). Our previous results showed that IL-37a and b can promote salmonella survival, probably by inhibiting TLR-induced proinflammatory cytokine and bactericidal NO production (Chapter 4). However, the molecular mechanism is still unknown. We therefore assessed the gene profile regulated by IL-37 isoforms on salmonella infection signalling pathway. As illustrated in Figure 5.16, the transcriptomic results agreed with the biological result in Chapter 4 and suggested that IL-37a and IL-37b also negatively regulate the key inflammatory genes, alone or together, in salmonella infection signalling pathways in splenocytes and macrophages. TLR4 signal in salmonella infection is mainly through the MyD88-dependent pathways, leading to the activation of AP1, P38 and NF-κB and inflammatory gene expression (Medzhitov, 2001; Toshchakov *et al.,* 2002). Both the IL-37a and IL-37b suppressed MyD88 and signalling molecules P38 and AP1. IL-37 isoforms also directly or indirectly suppressed the key pro-inflammatory cytokines and iNOs productions. Interestingly, we found that IL-37a also specifically inhibits the transcription of LBP, TLR4 and ERK1/2 in the pathway. The result may help to explain the results in Chapter 4 and suggests that the advanced regulatory effect of IL-37a in salmonella infection may be due to its capability of targeting more genes in the pathway than IL-37b (Figure 5.16).

While IL-37 promotes bacterial survival in this salmonella context, its ultimate effect on salmonella infection is still not fully understood. Inflammation induced by salmonella can be pathogenic and lethal. The anti-inflammatory effect performed by IL-37 in salmonella infection may be also beneficial to reduce the tissue damage and septic shock. It is worthwhile to further test the hypothesis by looking at the dual effect of IL-37 in lethal salmonella infection model.

I realised that the transcriptomic experiment has some limitations and could be improved. Whereas we have looked at the transcriptomic changes early (4 hours) and later (18 hours) after LPS stimulation, it will be ideal to have more LPS stimulation time points in splenocytes in order to see the kinetic changes in gene expressions. The important differentially expressed genes need to be further validated in protein levels by western blot, FACS or ELISA. Their function in IL-37-mediated effect also needs to be evaluated by siRNA knockdown or overexpression in immune cells. The transcriptomics study also shows that IL-37a and b may have global regulatory effect on other diseases beyond LPS-induced inflammation. Furthermore, the gene/pathways enhanced by IL-37a and/or b are less understood and could be potentially important in the understanding of the ultimate effect of the IL-37 isoforms in immunity and in disease. This will be discussed in the General discussion chapter and addressed in further studies.

Although the quality of our RNA-seq result is good and fits our hypothesis. We also have some shortages in these experiments which can be developed in later experiments: 1). The sample size of each group was not big enough. We only have three sample per group, which is the minimum number to do statistical analysis. For an ideal experiment. It will be better to enlarge the group size and test results from more time points for a clear change of the TLR stimulation related immune pathways. 2). The transgene expression (number of copies) can be furthered controlled by normalizing RNA level of these sample. The difference in transgene expression may largely affect our result trends. 3). To use a better control, we can use the primary macrophage from mice spleen instead of the RAW macrophage cell line, which can reflect the real condition in human.

In order to establish an ideal RNA-seq experiment based on our project. There are some strategies I would like to do for my future experiments: 1). Using primary macrophage instead of macrophage cell line, sorting macrophage from WT and Tg mice after Salmonella infection/ TLR stimulation by Facs then do sequencing. Since IL-37a is the dominate IL-37 isoform which expresses in the early stage, I would like to check the gene expression within 6 hours after infection. 2). Considering the limited experiment condition, we only did one TLR ligand stimulation (LPS). However, based on our previous results, other TLR ligands (such as IMQ and Fla-st)

also affect the gene expression of IL-37 and other cytokines production in human cells. Therefore, it is necessary to test whether IL-37a affect TLR relate pathways under other TLR ligands stimulation, and by which mechanism.

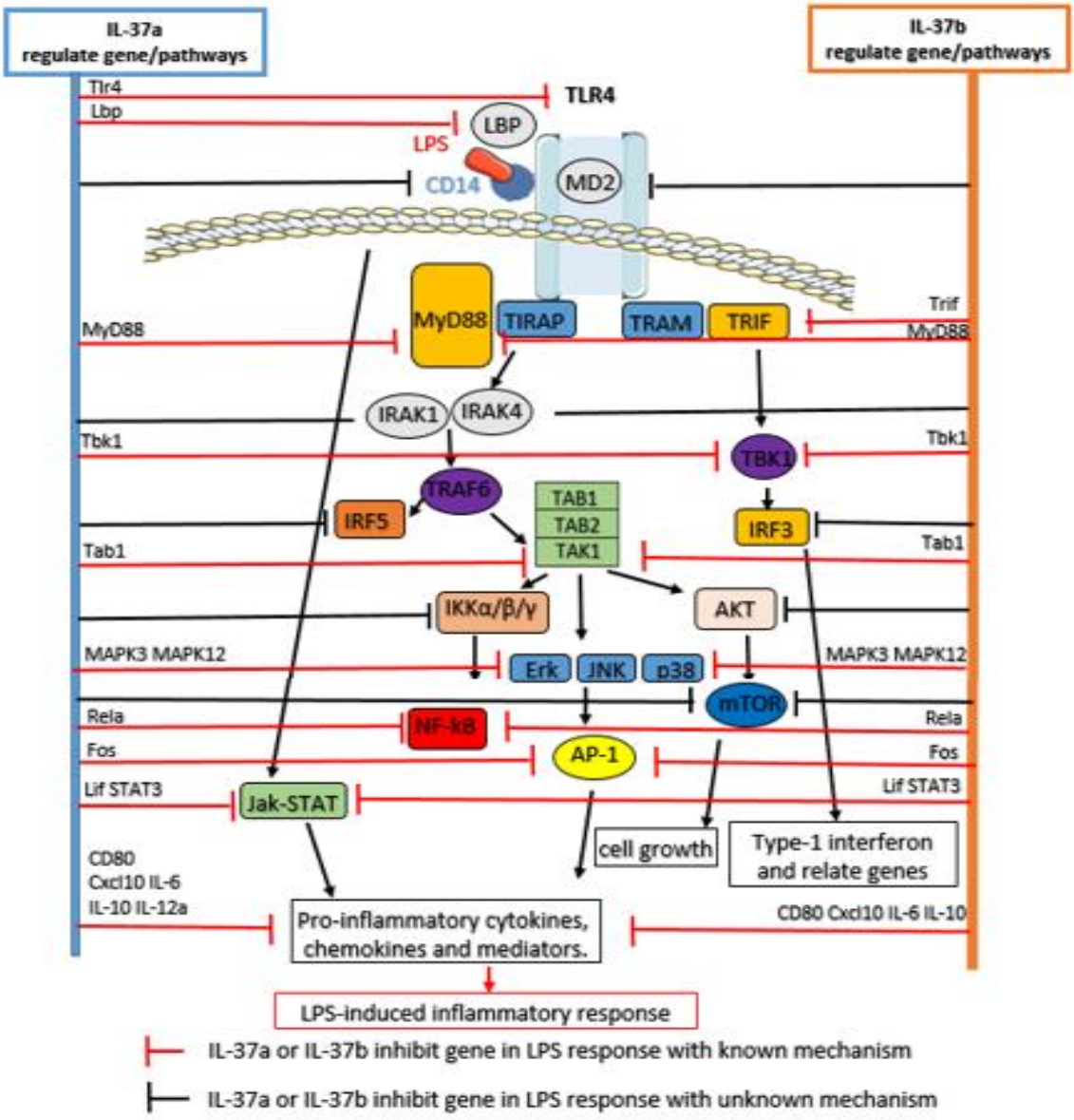



**Figure 5.15 Proposed molecular mechanism by which IL-37a and IL-37b regulated TLR4 signalling**

The genes related to TLR signalling pathway were analysed and generated in a proposed molecular pathway based on the Kyoto Encyclopedia of Genes and Genomes database (https://www.genome.jp/kegg/). Light blue represents the genes specific downregulated by IL-37a and light red represents the genes downregulated by IL-37b; blue/red represent genes regulated both by IL-37a and b. All the genes on this pathway are downregulated by IL-37a/b, there is no upregulation gene detected.

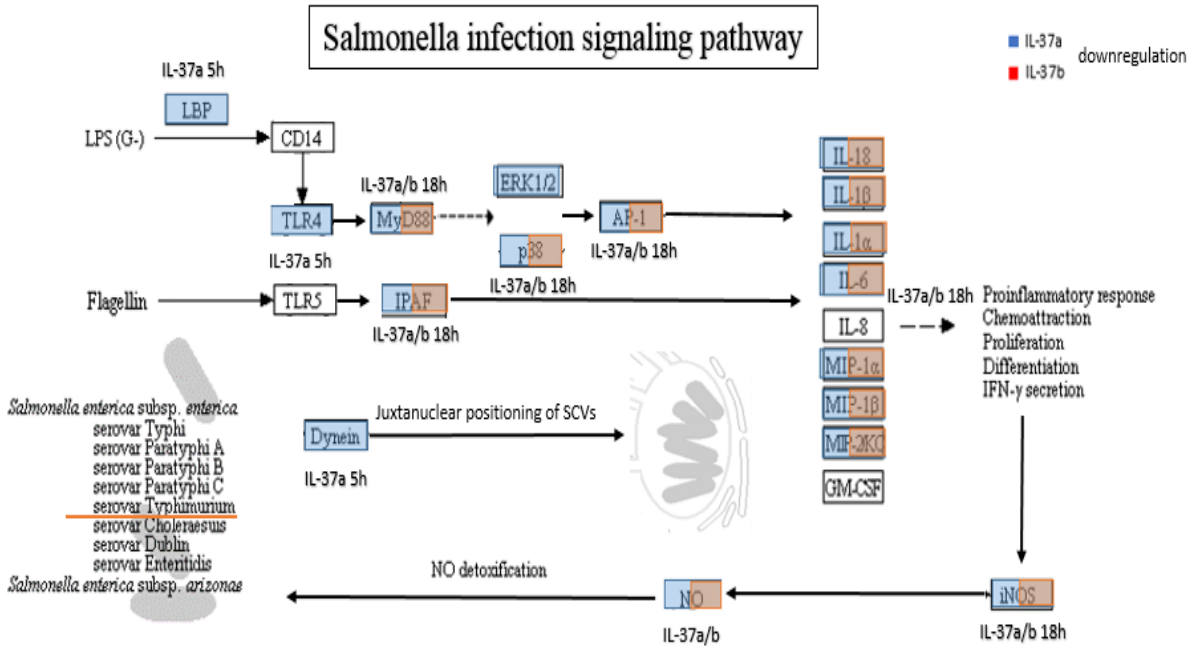



**Figure 5.16 Proposed molecular mechanism by which IL-37a and IL-37b regulated salmonella infection**

The genes related to salmonella infection were analysed and generated in a proposed molecular pathway based on the Kyoto Encyclopedia of Genes and Genomes database (https://www.genome.jp/kegg/). Light blue represents the genes specifically downregulated by IL-37a and light red represents the genes downregulated by IL-37b; blue/red represents gene regulate both by IL-37a and b. All the genes on this pathway are downregulated by IL-37a/b, there is no upregulation gene detected. (IPAF=NLRC4, Dynein=Dync)

# Chapter 6

# General discussion

# 6. General discussion

## 6.1 IL-37 isoforms: do we need 5 isoforms?

Interleukin-37 is a newly discovered member of the IL-1 superfamily, consisting of 5 isoforms (IL-37a-e) due to different RNA splicing. Current studies are only focused on IL-37b and show that IL-37b is an important immunosuppressive cytokine in inflammatory and autoimmune diseases. However, the biology and function of the other isoforms are still not clear. From the evolutionary perspective, the existence of a specific gene always has a necessary reason behind it; if a gene is no longer needed, it will be deleted or become a pseudo-gene. Therefore, it is important to understand the function of other IL-37 isoforms. Based on the previous studies mentioned in the general introduction of IL-37 and its isoforms (Chapter 1) and some result from our lab and our collaborators, in this chapter, I will mainly discuss the difference of IL-37 isoforms in their sequence, protein structure, induction, regulation, tissue distribution and potential function. In this case, we may have an answer for some questions of IL-37, at least, for the reason why we need all five isoforms.

### 6.1.1 IL-37 isoforms are different in gene organisation and protein levels

There are 6 exons of IL-37 gene which encode five isoforms of it with alternative mRNA splicing (Boraschi D *et al.,* 2011). To be specific, pro-IL-37a is a protein encoded by exon 3 to 6, which contains 192 amino acids. The pro-protein of IL-37a has a unique putative nuclear localization sequence (NLS) on the site of its N-terminal. NLS is a short sequence for importing of several nuclear proteins from cytoplasm into the cell nucleus by nuclear transport (Kalderon *et al.,* 1984). The pro-IL-37a also has a unique elastase cleavage which can be digested by elastase to become mature protein, while the rest of the IL-37 isoforms carry a caspase I cleavage site for protein maturation (Nold-Petry *et al.,* 2015).

The biggest isoform of the IL-37 family is IL-37b protein which contains 218 amino acids, this longest IL-37 isoform encoded by 5 of 6 total IL-37 exons, excepting exon3. The pro-IL-37b can be cleaved by caspase-1 to form mature IL-37b. The IL-37d protein is encoded by exon 1 and exons 4 to 6 to create a 197 amino acid long pro-protein and can also be processed by caspase I. Pro-IL-37c protein is 178 amino acid long which encoded by exons 1, 2, 5 and 6 and pro-IL-37e protein is 157 amino acid long which encoded by exons 1,5 and 6 (both of them lack exon 4). The most unique IL-37 isoform is IL-37a, which pro-protein is 192 amino acid long, encoded by exon 3 and exon 4 to 6; although IL-37a does not have exon 1 and 2, its exon3 encode a unique N-terminus to endow it with unique structure and function.

In these five isoforms, some of them contain an IL-1-like β-trefoil structure encoded by the exons 4-6, which is essential for receptor binding and signalling. According to the feature whether these isoforms carry the IL-1-like domain or not, the IL-37 isoforms can be further divided into two functional groups: 1) Isoform which has the IL-1-like domain, the IL-1-like β-trefoil structure encoded by the complete exon 4-6. The IL-37a, b and d have the complete domain which may be expected to bind to the same receptor, then trigger similar signal in immune response. And therefore, may have similar biological effects. 2) Isoform which lacks the IL-1-like domain. IL-37c and e do not have the exon 4 for their protein encoding, which may not have a partial IL-1-like β-trefoil structure. These isoforms may have different biological function from the former group whose function is still not clear. This evidence of isoform protein structure suggests that the isoforms of IL-37 may vary in their induction and function.

### 6.1.2 IL-37 isoforms differ in induction and expression

There is one possibility for the reason why we need all these five isoforms. These IL-37 isoforms may be selectively expressed in different organs or differently induced in different cell types or tissues in disease by various stimuli.

In fact, the IL-37 isoforms do have a tissue specific expression which has been studied (Taylor *et al.,* 2002). They also express in several common organs, like spleen and lymph nodes. To be specific, IL-37a is the only isoform which have expression in the brain; IL-37b is the only isoform which present in kidney; IL37c is specifically expressed in heart and IL-37d and e only have expression in bone marrow and testis, not in the other tissues. Therefore, IL-37 isoforms have a different tissue distribution and may have different function in variable cells and organs.

Current study also suggests that IL-37 gene expression is highly regulated under stimulation condition, e.g. LPS. There is an instability element (A-rich homology box) in exon 5 of IL-37 gene, which makes the IL-37 mRNA unstable (Bufler *et al.*, 2004). However, the function of this instability element of IL-37 gene can be overcome by the inflammatory signalling to ensure this gene can only be increased and expressed in inflammatory condition instead of in the homeostasis conditions. Moreover, resting cells can only produce IL-37b proteins in low level and this expression can be upregulated by several inflammatory stimuli and cytokines (TLR agonists, IL-1α, IL-18, TNF-α and IFN-γ) (Nold *et al.*, 2010). Therefore, it is important to explore the mechanism of how the other IL-37 isoforms are regulated and whether the regulation is acts in the same or different ways in immunity and disease. Since the information of this area is still very rare, mainly due to the potential importance of other IL-37 isoforms which has not been well studied.

There is only one report about other IL-37 isoforms except IL-37b determined the expression of all IL-37 isoforms in human PBMCs (Rudloff *et al.*, 2016). According to this result, the expression of IL-37 isoforms is differently regulated by several TLR agonists in PBMCs within 8 hours culture. The TLR agonist, LPS, can upregulate the expression of IL-37b and c in this experimental condition. Moreover, the expression of IL-37c is predominantly induced by Pam3, a TLR2 ligand. However, in this

experiment, the TLR7/8 ligands (IMQ) cannot influence the induction of IL-37 isoform expression. Furthermore, this report also revealed that the expression of IL- e cannot be induced by these stimulations at 8 hours.

This report was the first and only research on the regulation of IL-37 isoform expression in cell level to show a new insight for the different induction ways of IL-37 isoforms. However, there are some clear gaps in this research, for instance, this research only focuses on the gene express at 8 hours, which may not be long enough for gene expression, and the induction of IL-37 isoform expression beyond this time point has not been mentioned. Additionally, the detail sequence of qPCR primers for each IL-37 isoform was not provided in this study, which makes a bit difficult to interpret and repeat the real mRNA expression level of this result. Therefore, we designed specific primers for qPCR mainly based on their intron/exon bindery and further studied dose and time effect of TLR agonists on the expression of IL-37 isoforms. Our result indicates that the induction of IL-37 isoforms was both dose and time dependent (Chapter 4). Moreover, the result also shows that the IL-37a is one of the highly inducible IL-37 isoforms under TLR ligands stimulation in human macrophage cell lines (THP-1 cells) and primary cells (B cells, Najwa H, unpublished result).

### 6.1.3 Functional differences of IL-37 isoforms

Although the gene of IL-37 (IL1F7) has been identified for 19 years, its main biological function has only been confirmed recently. Moreover, only the biological function of isoform IL-37b and d has been studied. The function of the other isoforms is still unknown.

Current studies illustrate that the isoform IL-37b is a new cytokine belonging to the classical anti-inflammatory cytokine family which contains IL-10 and TGF-ß (Conti *et al.,* 2016). However, unlike multifunctional immunoregulatory cytokines, such as IL-10 and TGF-ß, the suppress function of IL-37 may only exist in the inflammatory response. This cytokine is inducible by the inflammatory signal and feedback to suppress the ongoing inflammatory response *in vitro* and *in vivo*.

IL-37b is thought to be a dual functional cytokine which can suppress inflammatory response via its receptor dependent and independent mechanisms (Dinarello *et al.,* 2016). In the receptor-dependent manner, the secreted IL-37b (mature or full-length) binds to receptor complex IL-18Ra/IL-1R8 (SIGIRR) then elitists anti-inflammatory signalling (Nold-Petry *et al.*, 2015). In the receptor-independent fashion, the mature but not the full-length IL-37b protein can interact with Smad3, a nuclear factor, which can translocate into cell nucleus and facilitate the cytokine-suppressing function in a Smad3-dependent mechanism. The importance and detail mechanism underlying the receptor-independent regulatory effect of IL-37b is still under investigation; while the protective effect of IL-37b against endotoxic shock induced LPS is receptor-dependent and needs the receptor complex IL-18Ra/IL-1R8 (SIGIRR) (Jia and Han, 2018).

Another IL-37 isoform, IL-37d, has been confirmed to be a bioactive cytokine recently (Zhao *et al.*, 2018). Similar to IL-37b, IL-37d can also bind to Smad3 and suppress inflammatory response via Smad3-dependent mechanism. However, different from IL-37b, the IL-37d cannot induce signal via the IL-37 co-receptor system. Since both IL-37b and d share the common C'- terminal sequence and the

IL-1 like domain, it is hard to understand why isoform IL-37d fails to signal via IL-37 co-receptors.

Since the function of isoform IL-37a had not been confirmed and its unique structure, this isoform is the current focus in our lab. Till now, we have found that the pro-protein of IL-37a works as a dual functional protein which can both act as a regulator in cell nuclear and also has cytokine function when secreted out by its producing cells (B cells and macrophages, Xu, unpublished result). Different from other isoforms of IL-37, the pro-IL-37a carries a unique putative NLS domain which may allow it to translocate into cell nucleus through this site.

Moreover, our result also confirms its nuclear location and further demonstrated that nuclear IL-37a can regulate other IL-37 isoform gene expression (Xu, unpublished result). Furthermore, our group also found that IL-37a possesses a C'-terminal structure which is similar to IL-37b and can also bind to the same receptor (IL-18Ra/IL-1R8) then trigger the similar signal and have similar biological effects. However, we expect that the unique structure of IL-37a may endow it a super regulatory function in immunity and disease in comparison with IL-37b and d. This hypothesis is currently under investigation in our lab and some preliminary results can support it.

There is no recent research about the function of the other two IL-37 c and e, which lack the exon4 and lose the full IL-1-like domain. Due to the existence of the 12 β-trefoils in the IL-1-like domain is essential for the receptor binding and signalling in IL-1 family; both IL-37c and e lack the first three β-strands of this structure, the IL-37c and IL-37e may not be able to bind to the co-receptor and therefore may lose the activity as cytokines (Nold *et al.,* 2010; Ellisdon *et al.,* 2017). However, these two IL-37 isoforms may work as a regulator to mediate the function of other IL-37 isoforms by competing or interfering with their mRNA splicing.

Because all the pro-IL-37 isoforms, apart from pro-IL-37a, contain a caspase I cleavage site and can be cleaved into mature protein by the same enzyme, IL-37c and IL-37e may also influence the maturation of other isoforms and their biological function by competing with the enzyme in the microenvironment *in vivo*. There is

an evidence to support this hypothesis: all cytokines in IL-1 family are self-regulated, for instance, IL-1α and ß can be regulated by their antagonist; IL-18 regulates via its binding protein (IL-18BP) and IL-33 regulates by the soluble IL-33 receptor (Hayakawa *et al.,* 2007; Dinarello *et al.,* 2013). On the other hand, although IL-37c and IL-37e fail to induce the receptor-dependent effect, they may also regulate gene expression via Smad3 to translocate into cell nuclear like IL-37b and Il-37d.

### 6.1.4 Conclusion and future directions

In summary, the five IL-37 isoforms are different in their protein structure, induction and function. There are three IL-37 isoforms (IL-37a, b and d) with the full-length IL-1-like domain and these have been demonstrated to play an essential immunoregulatory role in immunity and disease. These IL-37 isoforms may be therapeutically important in the treatment of many chronic disorders, especially in inflammatory and autoimmune diseases. Apart from these, many questions of IL-37 isoforms remain unknown: 1. Whether these isoforms have same or different function in regulating inflammations. 2. The precise mechanisms by which IL-37 isoforms suppress inflammatory response needs answer. 3. The variant participation and interaction of IL-37 isoforms in certain disease conditions are still not clear. 4.Although the first group of IL-37 isoforms which has the full-length IL-1-like domain is likely bioactive, the effect of IL-37c and IL-37e needs to be determined. 5. The importance and relationship of IL-37 with other immunoregulatory cytokines, such as IL-10 and TGF-b in immunotolerance need to be studied in the future.

Moreover, these IL-37 isoforms may synergise, back up or balance the function of each other in the regulation of inflammation. Therefore, the self-regulation of IL-37 ensures that the ongoing inflammatory response can be carefully and properly controlled.

## 6.2 IL-37: an immune inhibitor or regulator?

Il-37b has been initially identified as a fundamental inhibitor of innate immune response (Nold *et al.,* 2010). Following studies confirmed this hypothesis on innate immune cells and further revealed that IL-37 (IL-37b) can not only inhibits innate immune cells but also T-cells (Nold-Petry *et al.,* 2015). Thus, IL-37 may inhibit both innate and adaptive immunity. Moreover, all the current reports suggest that IL-37 is an inhibitor of immune system and can suppress immune cell functions via both direct and indirect mechanisms. However, current results from us and others suggest that IL-37 may also enhance immune cell function, such as enhance antibody production in B cells (Xu, unpublished result). Therefore, all evidence has raised an essential question: whether IL-37 is an immune inhibitor or a regulator. In the following part of my general discussion, I sought to answer the question by collecting and analysing existing results which support both proposals.

### 6.2.1 Evidence supporting IL-37 as an immune inhibitor

Much evidence supports IL-37 working as an immune inhibitor and confirmed that IL-37 can inhibit the function of both innate and adaptive immune cells. The IL-37 protein can be expressed in a broad range of immune cells, such as monocyte, macrophages, mast cells and T cells. Moreover, the expressed IL-37 protein plays an inhibitory role in these immune cell functions.

It has been demonstrated that monocytes express the highest level of IL-37 proteins compared with other immune cells in human (Rudloff *et al.,* 2016). Thus, human monocytes may be a main source of IL-37 protein production in immune response. It has also been reported that both mRNA and protein level of IL-37 are inducible in monocytes by mycobacterial tuberculosis in TB patients. Moreover, this expression is negatively correlated with the expression of several inflammatory cytokines, such as IL-1β, IL-6 and TNF-α (Zhang *et al.,* 2016). Although the detailed function of IL-37 on monocytes has not been studied, its function on macrophages has been studied in many research centres, including our lab. IL-37 can effectively suppress

inflammatory cytokine production induced by TLR signalling (Nold-Petry *et al.*, 2015; Huang *et al.*, 2016). Furthermore, IL-37b can effectively suppress the production of TNF-α, IFN-γ and NO in human macrophage under oxygenated low-density lipoprotein (ox-LDL)-activation condition (Huang *et al.*, 2016).

Mast cell acts as a pathogenic cell in many disease conditions, such as allergy, asthma and psoriasis (Conti, 2017). Some studies demonstrate that IL-37b protein can suppress mast cell migration and its function in murine disease model of psoriasis (Teng *et al.*, 2014) and asthma (Conti *et al.*, 2016).

Moreover, the effect of IL-37b on adaptive immune cells has been assessed and only the influence of T cells has been confirmed till now. The report illustrate that the IL-37 protein can be expressed by CD3+ and CD4+ T cells which can further inhibit the function of Th 17 cells in human (Ye *et al.*, 2014). In the situation of Th17 cell polarisation, the expression of IL-37 can significantly inhibit IL-17 expression and the proliferation of Th17 cells but cannot affect their differentiation from naive human CD4+ T cells *(ibid)*.

Unlike the well-studied isoform IL-37b, we are in the initial stage to explore the regulatory effect of IL-37a and IL-37d. The only report illustrates that IL-37d is expressed in human peripheral blood mononuclear cells (PBMCs), umbilical cord-derived mesenchymal stem cells (UCMSCs) and epithelia. The overexpression of IL-37d inhibits inflammatory cytokine IL-6 production which is induced by IL-1b in macrophages and epithelia cell line (Zhao *et al.*, 2018). However, the regulation function of IL-37d in the other immune cells has not been studied to date. Furthermore, our results confirmed that isoform IL-37a is another bioactive IL-37 isoform like IL-37b, which can suppress the inflammatory cytokine production (IL-6. IL-1 and TNF) induced by LPS stimulation in human and murine macrophage and monocytes (Chapter 4; Xu, unpublished results). Its effect on other innate and adaptive immune cell function is currently under investigation. Although the exact mechanisms of how IL-37 inhibits these cells are still poorly understood, current evidence shows that IL-37 can suppress innate and adaptive immune cell function in a direct or indirect way.

#### 6.2.1.1 The direct inhibitory effect of IL-37 on immune cell activity

The direct inhibitory effect of IL-37 protein is performed in autocrine or paracrine manner. Through the autocrine pathway, IL-37 will be initially produced by an immune cell, followed by cytokine secretion, then have the regulatory function via receptor dependent or independent pathways. As discussed in the first part of general discussion, IL-37a, b and d can translocate into cell nuclear then work as a nuclear factor to have their regulatory function without the assistance of the receptors. However, the mechanism of how nucleus IL-37 isoforms regulate pro-inflammatory cytokine gene function remains unknown. Nevertheless, the receptor-dependent regulatory effects of IL-37 isoforms are comparatively better studied; IL-37a and b protein, apart from IL-37d, can bind to their common co-receptors and have their regulatory function by receptor-mediated signalling (Nold-Petry *et al.*, 2015; Zhao *et al.*, 2018). This phenomenon was confirmed in murine model by the lost inhibitory function of endogenous or recombinant IL-37 (IL-37b) in IL-37 receptor knockout (IL-1R8KO) mice or mice treated with anti-IL-37 receptor antibody (Nold-Petry *et al.*, 2015).

Through the paracrine pathway, the IL-37 will be initially produced by some types of immune cells, for instance, monocytes from human donor can suppress the function of other immune cells which is mainly possess by IL-37 receptor (IL1R8) (Zhao *et al.*, 2018). Therefore, it allows the enlargement of IL-37-regulated function in immune response in tissue and organ.

#### 6.2.1.2 The indirect inhibitory effect of IL-37 on immune cell activity

Additionally, IL-37 may further regulatory immune activation in an indirect way by suppressing Antigen-presenting cell (APC) function and/or by inducing Regulatory T cells (Treg) and tolerogenic dendritic cells (tDC) (Nold-Petry e*t al.*, 2015). Therefore, this could cause a systemic influence of IL-37 on the ongoing inflammatory response.

IL-37 effect on APC function: DC is an essential APC in tissues which act as a crucial factor in the initiation of immune response. Results show that the IL-37 protein can prevent the maturation and antigen presentation of DCs. For instance, the maturation and activation of DCs are induced by LPS, which shows a significant reduction in IL-37bTg mice compared with WT (Luo *et al.,* 2014). The similar result can be observed in IL-37 treated DCs, which show a decreased expression of MHC II and the costimulatory molecule CD40 then reduced production of inflammatory cytokines, such as IL-1ß, IL-6, and IL-12 (Luo *et al.,* 2014). Moreover, IL-37b-treated DC cannot effectively activate naive T cells (Chang *et al.*, 2017). Therefore, the regulatory function of IL-37b may influence the development of antigen-specific immune response. Furthermore, the potential effect of other IL-37 isoforms in the regulation of APC, Treg and tDC is still unknown. The influence of IL-37 on tDC and Treg development and their function will be discussed as below.

#### 6.2.1.3 IL-37 can enhance function of immune cell sets

The inhibitory function of IL-37 in immunity and disease has been well studied, the immune enhancement effect of IL-37 has been largely neglected. According to the existing review and analysis of the results from our and other labs, some evidence supports that IL-37 can also efficiently promote functions of some immune cells.

Treg cells, especially the Forkhead box proteins-3 (FOXP3+) Treg, are fundamental cell types in the regulation of development of inflammatory and autoimmune disease (Long and Buckner, 2011). It has been reported that IL-37 can also be expressed by T cells which in turn differently regulate the function of CD4+ T cell subsets. The treatment of recombinant IL-37 protein can selectively decrease Th1 and Th17 cell development but enhance Treg cell function and polarisation (Ji *et al.,* 2017). Moreover, IL-37 also expresses in CD4+ Treg which promotes the expression of Treg transcript factor FOXP3; it also enhances the expression of suppressive cytokines such as IL-10 and TGF-ß in Treg (Shuai *et al.,* 2015). Furthermore, silencing the IL-37 gene in human Treg impairs the suppressive function of Treg (Shuai *et al.,* 2015).

IL-37 may also regulate inflammatory response through the induction of tolerogenic DC (tDC). The tDC is a DC subset which acts as a regulatory cell in immune tolerance. Different from other DC, tDC is characterised by its ability of immune suppression and the polarisation of Treg (Chang *et al.*, 2017). It has been reported that the DCs isolated from IL-37bTg mice express IL-10 in high level and reduced their function in the antigen-specific T cells activation, which suggests that these DCs are the tDCs. Moreover, the tDC can polarise Treg cells and decrease the CD8+ cytotoxic function *in vitro* and in contact hypersensitivity of mice (Luo *et al.*, 2014). Thus, by inhibiting APC and promoting development and functions of Treg and tDC, IL-37 can maximise its systemic regulatory function in order to control inflammatory response more effectively. However, these studies only focus on IL-37b, the potential effect of other IL-37 isoforms in the regulation of immune cells is still unknown.

IL-37 also plays a role in regulating macrophage functions. Macrophages (M0) can be further polarised into M1 or M2 subtypes (Martinez and Gordon, 2014). The M1 macrophages are essential in infection and inflammation. These cells are characterised by the expression of iNOs and NO production, and expression of several pro-inflammatory cytokines, including IL-1b and IL-12 (Castleberry *et al.*, 2008). The M2 macrophages, on the other hand, play an important role in immunoregulation and tissue repair by producing suppressive cytokines IL-10, TGF-β, arginase 1 (ARG1) and suppressing NO production. It has been demonstrated that IL-37 can enhance the development of M2 macrophages in mycobacterium tuberculous infection by elevating the expression of ARG1, IL-10, TGF-β and reducing NO production in the macrophages (Huang *et al.*, 2015). Furthermore, IL-37b also effectively suppresses the ox-LDL-induced M1 macrophage development, which in turn polarise these cells into M2 cells (Huang *et al.*, 2016). These evidences demonstrate that IL-37 can selectively polarise macrophages into M2 cells. Therefore, IL-37 may acts as IL-10 and TGF-B which participate in tissue repair and homeostasis.

### 6.2.2 Transcriptomic evidence supports that IL-37 can enhance immune response

The transcriptomic (RNA sequencing) results from our own and other labs also demonstrated the enhancement function of IL-37 in the transcriptome in immune cells. One report illustrates that IL-37b can predominantly upregulate the expression of many genes, while it can also downregulate many gene expressions in the LPS-stimulated splenocytes from IL-37b transgenic mice (Nold-Petry *et al.,* 2015). This result suggests that IL-37b may work in both aspects, which can either to enhance or suppress gene expressions in the LPS-stimulated cells. Unfortunately, the value and related signal pathways of these enhanced genes have not been provided in this article. Instead, the authors focused on the 139 genes which associate with innate immune response and showed that 46% of them were enhanced by IL-37b in the context. With further analysis of these genes which upregulate by IL-37b, they found that these genes belong to two groups: 1. Some of these genes encoded inhibitors of inflammation. 2. Others are closely associated with inflammatory response, for instance, the TRAIP (a TLR adaptor), Plcd3, TNF-α, S100a (a TLR4 agonist) and Pycard gene (Nold-Petry *et al.,* 2015).

However, the immunological significance of these upregulated inflammatory genes in IL-37-mediated effect in the immune context has not been well studied. Nevertheless, these results demonstrated that IL-37b not only inhibits but also enhances some gene expression in immune cells.

## 6.3 Role of IL-37 in infectious diseases, friend or foe?

Accumulating reports suggest that IL-37 plays a critical, but controversial role in infectious disorders, depending on pathogen (Wang *et al.,* 2018; Allama *et al.,* 2019). Thus, it is important to be fully aware of the dual effects of IL-37 on the development and pathogenesis of infectious diseases. Here I will give a general view of each type of infectious disease first, including virus, bacterial, helminth and fungi infection, and then focus on the critically studied IL-37-regulated diseases in this category to assess the impact of IL-37 on this type of disease. I hope the review will

help to understand the potential beneficial and detrimental effect of IL-37 on infectious disease, including salmonellosis for better clinical application.

The implication of IL-37 in infectious disorders is poorly understood, particularly in the parasite infection, and likely to be complicated, depending on the pathogen. IL-37b exhibits a profound effect on the development and perpetuation of a wide range of infectious diseases, including virus, bacterial, parasite and fungi infection. However, the effect of IL-37 on diseases is varying, and can be beneficial or detrimental.

In virus diseases tested, IL-37b is generally protective in Influenza and coxsackie virus B3 (CVB3) infection. The IL-37-mediated beneficial effect was largely attributed to its role in the downregulation of virus-induced inflammatory response. Intriguingly, IL-37 can also directly suppress the replication of influenza virus in epithelia cells. Thus, IL-37b may be potentially therapeutic in the treatment of virus infections.

The role of IL-37 in mycobacterium tuberculosis (Mtb) and Streptococcus pneumonia infection is controversial, depending on the bacterial and immune context, and could be beneficial or detrimental. This certainly is the case in Mtb infection in mice and in human. More studies are needed to clarify the issue.

In Streptococcus pneumonia (SP) infections, IL-37 appears to be pathogenic by harnessing the protective inflammatory response and promoting bacterial growth. Thus, IL-37 is a potential therapeutic target molecule.

Similar to SP infection, IL-37 also promotes candida infection, enhanced fungal growth in the organ and mortality. IL-37b does so also by downregulating the protective inflammatory cytokine produces and the recruitment of neutrophils to the site of infection. In contrast, IL-37-reduced inflammatory response and increased IL-10 production in lung tissue during aspergillosis is protective against aspergillus infection. Thus, it is clinically important to understand the contradictory role of IL-37-medidated anti-inflammatory effect in the outcome to bacterial infection.

Finally, the impact of IL-37 on parasite infection is less studied and largely unknown. Given the clinical importance of parasite infection in human health, more attention should be paid to this ignored area.

Thus, IL-37 may have impact on the treatment and prognostic of infectious diseases, dependent on type of pathogen. Future studies should focus on the systemic effect of IL-37 on homeostasis and immunity in infectious disorders. While the basic research from animal models has greatly enhanced our knowledge of the potential application of IL-37 in clinical diseases, its importance in human disease is less understood. The function and relation of other IL-37 isoforms, including IL-37a in human diseases is still largely unknown. Since IL-37 is selectively expressed in human but not mice it will be interesting to see whether this can influence host resistance/susceptibility to infection in mice and human.

## 6.4 What is the impact of IL-37a on immunoregulation of inflammation?

The immune response is tightly controlled by multiple mechanisms including regulatory cytokines (Murphy and Weaver, 2016). Dysregulation of any step of the initiation, propagation and resolution of the immune response will increase the risk of developing an inflammatory disorder.

An inflammatory response can be initiated by antigen-dependent or independent mechanisms. In the former, an inflammatory response can be initiated by a dysregulated antigen-presenting cell (APC) and/or dysregulated adaptive immune cell function. In the latter, physical (e.g. injury or heat), chemical or biological factors (e.g. toxins, PAMPs) can damage host tissue. This damage will initiate the release of alarmins and DAMPs, for example, inflammatory cytokines IL-1 and IL-33 in innate immune cells located in the damaged tissue (Abbes *et al.*, 2011). The released DAMPs and PAMPs will then activate more innate immune cells that expressing the appropriate receptors, for example, macrophages, neutrophil and mast cells, and will induce these cells to produce more inflammatory cytokines which will amplify the acute inflammatory response (Tang *et al.*, 2012). In both contexts, if the innate and adaptive immune response is intense and/or persistent,

it may cause tissue damage and death, for example, LPS-induced shock in infectious diseases and autoimmune disorders respectively.

While the detailed mechanism by which IL-37a regulates the inflammatory response is still largely unknown, our early results suggest that IL-37a may play an important role in the regulation of key steps of innate and adaptive inflammatory response.

This new knowledge has improved our understanding of the immunoregulation of inflammation in several aspects as follows:

### 6.4.1 IL-37a governs ongoing innate and adaptive inflammatory responses in a negative immunoregulatory feedback-loop

Until now the relative induction and expression of different IL-37 isoforms in immune cells were unknown. We demonstrated that IL-37a is the predominant induced isoform in TLR-stimulated human PBMCs. We also found that IL-37a is widely expressed by most innate immune cells, including macrophages, monocytes, and DCs (Xu, data not shown). Following LPS stimulation, we demonstrated that monocytes express highest levels of IL-37 mRNA, including IL-37a among the immune cells, therefore monocytes may represent the main source of IL-37 in humans. Tissue cells, fibroblasts and epithelial cells also express IL-37a mRNA, suggesting that local IL-37a may contribute to regulation of the inflammatory response in stromal tissue. Furthermore, resting immune cells express constitutively low levels of IL-37a and this expression can be markedly enhanced by inflammatory mediators, for example, by TLR ligands and inflammatory cytokines IL-1 and TNFα. This is partly because the IL-37 mRNA contains an instability element which can be stabilised by inflammatory signals (Bufler *et al.*, 2004).

The impact of IL-37a on the adaptive immune response is less well understood and is currently under investigation. Our early results indicate that T and B cells express IL-37a, and that importantly, IL-37a and IL-37b can regulate DC, T and B cell functions. It has been shown that IL-37b plays an important role in the regulation of adaptive immune responses by interfering DC antigen presentation and Th1 and Th17 cell polarisation and function in inflammatory and autoimmune diseases (Nold-Petry *et al.*, 2015; Boraschi *et al.*, 2011). Since IL-37a and IL-37b signal via the same

receptor it is anticipated that IL-37a might have same effects as IL-37b. It is already known that the IL-37 receptor complex, IL-18R and IL-1R8 is expressed by almost all innate and adaptive immune cells (Lech *et al.,* 2007). However, the selective expression and location of IL-37 isoforms in human tissues is largely unknown, mainly due to the lack of specific antibodies for tissue staining by immunohistochemistry.

Thus, IL-37a can directly and indirectly affect innate and adaptive immune cell function in autocrine and paracrine fashions.

Since IL-37a is induced by inflammatory factors and hinders ongoing inflammatory responses, IL-37a may have evolved to restrain aberrant hyper-reactivity, but not to interfere with normal immune activation. Therefore, IL-37a may be an early checkpoint of the inflammatory response.

### 6.4.2 IL-37a can regulate the inflammatory response by IL-1R8-dependent and independent mechanisms

We demonstrated that recombinant IL-37a inhibits the in vitro production of inflammatory cytokines IL-1 and TNFα by LPS-stimulated splenocytes from WT but not IL-1R8-/- mice. This suggested that IL-37a and IL-37b shared the same functional receptor because both isoforms contain the IL-1-like domain for receptor binding and signalling. Further experiments are needed to study in detail how IL-37a signals via IL-1R8 and whether the co-receptor IL-18R is required for signalling.

A striking feature of IL-37a is its nuclear localisation via its NLS; no other IL-37 isoforms has the capability. I have not studied the nuclear function of IL-37a as part of my thesis, others in my research team have found that nuclear IL-37a can interact with several nuclear proteins and can regulate inflammatory response by receptor-independent mechanism, for example, the N-terminal IL-37a in the nuclear can suppress MAPK13 and TNFα (Xu, D *et al*, unpublished result). Thus, IL-37a is a dual functional immunoregulatory factor that can control inflammatory responses via receptor dependent and independent pathways.

### 6.4.3 IL-37a can help identify different human and mouse immunoregulation systems

Since IL-37 is expressed in humans but not in mice, a comparative study of IL-37 function will help to understand different mechanisms by which mice and humans regulate inflammatory disease. This is an important issue because the mouse has been widely used as an exploratory model for many human immune responses and diseases. Whereas the immunological meaning of the differential requirement of IL-37 in the human and mouse remains unclear, experimental results from our and other group (Nold-petry *et al.*, 2015) suggest that the selective expression of IL-37 makes the human immune system more manageable than the mouse. This may contribute to better regulation of inflammatory diseases in humans. However, we found evidence for more subtle complexity in the role of IL-37. In a salmonella infection model we founds that transgenic expression of IL-37a in mice renders them less resistant to salmonella infection. We also found that this was in part due to IL-37 inhibition of microbicidal nitric oxide and inflammatory cytokine production. This suggests that the selective expression of IL-37a may make human more susceptible and mouse resistant to infection, and that evolutionary loss of IL-37 in the wild may help survival. The relative function of IL-37 in humans and mice may need to be considered in the limitation of mouse model in the study of mechanisms of infection and perhaps other human immune-mediated disease that involves IL-37.

### 6.4.4 IL-37 isoforms act as a composite regulatory system in which IL-37a has a dominant role.

Among the five IL-37 isoforms (IL-37a-e), the three isoforms (IL-37a, b and d) with an intact IL-1-like domain are found to have immunosuppressive function. Therefore, these isoforms may represent a novel immunoregulatory checkpoint to ensure the inflammatory response is under control.

It is intriguing to better understand the functional differences between the isoforms, and their relative importance in immunoregulation. During my thesis I found that IL-37a and b are the most responsive to induction by TLR ligands in human PBMCs, and that IL-37a is more immunosuppressive than IL-37b in TLR response *in vitro* and *in vivo*. This is mainly attributed to the additional regulatory effect of nuclear IL-37a. Given that among the isoforms, IL-37b and d share greatest similarity in protein

sequence and function, it is likely IL-37a may be the dominant isoform in IL-37-mediated effects. Future investigations, in particularly in diseases will confirm this hypothesis.

The difference and interaction between IL-37 and other known regulatory cytokines, including IL-10 and TGFβ has not been fully investigated. Current evidence suggests that IL-10 and TGFβ are pleotropic cytokines that can inhibit or enhance certain type of immune responses. In contrast, IL-37 seems to be predominantly immunosuppressive. Furthermore, IL-37-mediated immunosuppression is independent of IL-10 and TGFβ, and IL-37 can inhibit IL-10 and TGFβ function, for example in psoriasis and fibrosis, respectively (Teng *et al.*, 2014; Kim *et al.*, 2019). More studies are needed to understand their inter-relationship in immunoregulation.

## 6.5 Future directions for study

The study of IL-37a is in its infancy; there are many more questions than answers.

For my future research, I will undertake the following projects:

1. **Difference between human and mice in Salmonella infection - role of IL-37a**

I have discussed with my supervisor Dr Xu and the following experiments to complete the current project and produce a publication.

- Human study: Collect blood samples from healthy donors and patients with salmonella infection and determine their difference in IL-37 isoforms, inflammatory cytokines and iNOS expression in PBMC by qPCR and mediator concentrations in serum by ELISA. I would predict here that the Salmonella infected patients may have higher IL-37 gene expression and protein expression, which may lead to the downregulation of pro-inflammatory cytokine expression. However, the IL-37 level may also regulate by some unknow mechanism which protect patients from bacteria burden.

  Confirm the difference between human and mouse in salmonella infection in primary macrophages *in vitro*.

In vivo study: We sought to using WT *Salmonella typhimurium* strain to further confirm the IL-37 effect in attenuate salmonella infection. This is a real infection model and allows to observe the protective effect of IL-37 on salmonella-induced mortality.

In order to reveal receptor-dependency of the IL-37a mediated effect on salmonella infection, the experiment can also be done in an IL-37aTg/IL-1R8-/- mouse strain which is now available.

Immune mechanism study: the regulatory role of IL-37a on adaptive immune response is less well understood. Since a Th1 response is required for the resolution of salmonella infection, the potential role of IL-37a in DC function, Th1 development, migration and function will be studied.

Molecular mechanism: how IL-37a inhibits NO expression is unknown and will be studied in detail. If IL-37a affects Th1 development and/or function, the molecular mechanism will also be revealed.

Together, these experiments and the findings in my thesis will provide a fuller picture on how IL-37a regulates salmonella infection in human and in transgenic mice.

**2. Potential therapeutic effect of IL-37a in inflammatory disorders:**

We and others have demonstrated in vitro and in vivo the immunosuppressive effects of IL-37a and b, respectively, on inflammatory response development by using transgenic mice, virus vector and recombinant protein. While the preventive effect is important, there is no convincing evidence that IL-37 has a therapeutic effect in disease models in vivo using current approaches. This is mainly because the natural IL-37 protein exists in a monomeric form and only the monomer but not the homodimer is bioactive and current recombinant IL-37 proteins are largely homodimer. By using point mutation of the amino acid involved in the dimerization, we have successfully produced recombinant IL-37a monomers that are substantially more suppressive than the dimer in in vitro assay (Chapter 3). The recombinant IL-37a monomer proteins will be used to test its therapeutic potential in a variety of infectious and autoimmune disease models, including salmonella infection, collagen induced arthritis and psoriasis in mice. If IL-37a is therapeutic and safe, its application in human diseases might be considered.

## References


1. Abbas, A., Lichtman, A. and Pillai, S. (2012). Cellular and molecular immunology. Philadelphia: *Elsevier*,

2. Aderem, A. and Underhill, D. (1999). Mechanisms of Phagocytosis In Macrophages. *Annual Review of Immunology*, 17(1), pp.593-623.

3. Akira, S., Uematsu, S., Takeuchi, O. (2006). Pathogen recognition and innate immunity.*Cell,* 124(4), PP. 783–801

4. Alber, A., Howie, S., Wallace, W. and Hirani, N. (2012). The role of macrophages in healing the wounded lung. *International Journal of Experimental Pathology,* 93(4), pp.243-251.

5. Allam, G., Gaber, A.M., Othman, S.I. and Abdel-Moneim, A., 2019. The potential role of interleukin-37 in infectious diseases. *International reviews of immunology*, pp.1-8.

6. An, B., Liu, X., Li, G. and Yuan, H., 2017. Interleukin-37 ameliorates coxsackievirus B3-induced viral myocarditis by modulating the Th17/regulatory T cell immune response. *Journal of cardiovascular pharmacology*, *69*(5), pp.305-313.

7. Anuradha, R., Munisankar, S., Bhootra, Y., Dolla, C., Kumaran, P., Nutman, T.B. and Babu, S., 2017. Modulation of CD4+ and CD8+ T cell function and cytokine responses in Strongyloides stercoralis infection by interleukin-27 (IL-27) and IL-37. *Infection and immunity*, *85*(11), pp.e00500-17.

8. Aoki-Kinoshita, K.F. and Kanehisa, M., 2007. Gene annotation and pathway mapping in KEGG. In *Comparative Genomics* (pp. 71-91). Humana Press.

9. Arndt, C., Koristka, S., Bartsch, H. and Bachmann, M., 2012. Native polyacrylamide gels. In *Protein electrophoresis* (pp. 49-53). Humana Press, Totowa, NJ.

10. Arulraj, T. and Barik, D., 2018. Mathematical modeling identifies Lck as a potential mediator for PD-1 induced inhibition of early TCR signaling. *PloS one*, *13*(10), p.e0206232.

11. Bajpai, V., Baek, K. and Kang, S. (2012). Control of Salmonella in foods by using essential oils: A review. Food Research International, 45(2), pp.722-734.

12. Ballak, D.B., Van Diepen, J.A., Moschen, A.R., Jansen, H.J., Hijmans, A., Groenhof, G.J., Leenders, F., Bufler, P., Boekschoten, M.V., Müller, M. and Kersten, S., 2014. IL-37 protects against obesity-induced inflammation and insulin resistance. *Nature communications*, *5*, p.4711.

13. Berry, M.P.R., Blankley, S., Graham, C.M., Bloom, C.I. and O'Garra, A., 2013. Systems approaches to studying the immune response in tuberculosis. *Current opinion in immunology*, *25*(5), pp.579-587.

14. Beutler, B., Jiang, Z., Georgel, P., Crozat, K., Croker, B., Rutschmann, S., Du, X. and Hoebe, K., 2006. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annu. Rev. Immunol.*, *24*, pp.353-389.

15. Bollag, D.M. and Edelstein, S.J., 1991. Protein methods. Wildy-Liss. *Inc., New York, NY*.

16. Boraschi, D., Lucchesi, D., Hainzl, S., Leitner, M., Maier, E., Mangelberger, D., Oostingh, G.J., Pfaller, T., Pixner, C., Posselt, G. and Italiani, P., 2011. IL-37: a new anti-inflammatory cytokine of the IL-1 family. *European cytokine network*, *22*(3), pp.127-147.

17. Bouali, E., Kaabachi, W., Hamzaoui, A. and Hamzaoui, K., 2015. Interleukin-37 expression is decreased in Behçet's disease and is associated with inflammation. *Immunology letters*, *167*(2), pp.87-94.

18. Breuer, K., Foroushani, A., Laird, M., Chen, C., Sribnaia, A., Lo, R., Winsor, G., Hancock, R., Brinkman, F. and Lynn, D. (2012). InnateDB: systems biology of innate immunity and beyond—recent updates and continuing curation. *Nucleic Acids Research,* 41(D1), pp.D1228-D1233.

19. Bufler, P., Gamboni-Robertson, F., Tania, A.Z.A.M., Soo-Hyun, K.I.M. and Dinarello, C.A., 2004. Interleukin-1 homologues IL-1F7b and IL-18 contain functional mRNA instability elements within the coding region responsive to lipopolysaccharide. *Biochemical Journal*, *381*(2), pp.503-510.

20. Bulau, A.M., Nold, M.F., Li, S., Nold-Petry, C.A., Fink, M., Mansell, A., Schwerd, T., Hong, J., Rubartelli, A., Dinarello, C.A. and Bufler, P., 2014. Role of caspase-1 in nuclear translocation of IL-37, release of the cytokine, and IL-37 inhibition of innate immune responses. *Proceedings of the National Academy of Sciences*, *111*(7), pp.2650-2655.

21. Busfield, S.J., Comrack, C.A., Yu, G., Chickering, T.W., Smutko, J.S., Zhou, H., Leiby, K.R., Holmgren, L.M., Gearing, D.P. and Pan, Y., 2000. Identification and gene organization of three novel members of the IL-1 family on human chromosome 2. *Genomics*, *66*(2), pp.213-216.

22. Carruth, L.M., Demczuk, S. and Mizel, S.B., 1991. Involvement of a calpain-like protease in the processing of the murine interleukin 1 alpha precursor. *Journal of Biological Chemistry*, *266*(19), pp.12162-12167.

23. Carson, M., Johnson, D.H., McDonald, H., Brouillette, C. and DeLucas, L.J., 2007. His-tag impact on structure. *Acta Crystallographica Section D: Biological Crystallography*, *63*(3), pp.295-301.

24. Casals, C., Barrachina, M., Serra, M., Lloberas, J. and Celada, A. (2007). Lipopolysaccharide Up-Regulates MHC Class II Expression on Dendritic Cells through an AP-1 Enhancer without Affecting the Levels of CIITA. *The Journal of Immunology,* 178(10), pp.6307-6315.

25. Castleberry, J., Scott, A., Alusta, P., Soderberg, L. and Tarasenko, O. (2008). P7. Nitric oxide involved in macrophage activation and neutralization of Bacillus anthracis toxins during glycoconjugate treatment. *Nitric Oxide,* 19, p.1.

26. CDC. FoodNet 2011 Surveillance Report.

27. Chang, K., Song, J.Y. and Lim, D.S., 2017. Tolerogenic dendritic cell-based immunotherapy. *Oncotarget*, *8*(53), p.90630.

28. Charrad, R., Berraïes, A., Hamdi, B., Ammar, J., Hamzaoui, K. and Hamzaoui, A. (2016). Anti-inflammatory activity of IL-37 in asthmatic children: Correlation with inflammatory cytokines TNF-α, IL-ß, IL-6 and IL-17A. *Immunobiology,* 221(2), pp.182-187.

29. Chen, W., 2011. Tregs in immunotherapy: opportunities and challenges. *Immunotherapy*, 3(8), pp.911-914.

30. Crotty, S., 2014. T follicular helper cell differentiation, function, and roles in disease. *Immunity*, 41(4), pp.529-542.

31. Coburn, B., Grassl, G.A. and Finlay, B.B., 2007. Salmonella, the host and disease: a brief review. *Immunology and cell biology*, *85*(2), pp.112-118.

32. Conesa, A., Madrigal, P., Tarazona, S., Gomez-Cabrero, D., Cervera, A., McPherson, A., Szcześniak, M.W., Gaffney, D.J., Elo, L.L., Zhang, X. and Mortazavi, A., 2016. A survey of best practices for RNA-seq data analysis. *Genome biology*, *17*(1), p.13.

33. Conti, P. (2017). Possible role of mast cells and IL-37 in the pathogenesis of psoriasis. *Clinical Dermatology,* 5(1), p.30.

34. Conti, P., Ronconi, G., Caraffa, A., Lessiani, G. and Duraisamy, K. (2016). IL-37 a New IL-1 Family Member Emerges as a Key Suppressor of Asthma Mediated by Mast Cells. *Immunological Investigations,* 46(3), pp.239-250.

35. Corliss, B.A., Azimi, M.S., Munson, J.M., Peirce, S.M. and Murfee, W.L., 2016. Macrophages: an inflammatory link between angiogenesis and lymphangiogenesis. *Microcirculation*, *23*(2), pp.95-121.

36. Crump, J.A., Luby, S.P. and Mintz, E.D., 2004. The global burden of typhoid fever. *Bulletin of the World Health Organization*, *82*, pp.346-353.

37. de Sousa, J.R., Prudente, R.L., Junior, L.B.D., Carneiro, F.R.O., Sotto, M.N. and Quaresma, J.A.S., 2018. IL-37 and leprosy: A novel cytokine involved in the host response to Mycobacterium leprae infection. *Cytokine*, *106*, pp.89-94.

38. Delves, P. and Roitt, I. (2011). Roitt's essential immunology. Chichester, West Sussex: *Wiley-Blackwell*.

39. Demain, A.L. and Vaishnav, P., 2009. Production of recombinant proteins by microbes and higher organisms. *Biotechnology advances*, *27*(3), pp.297-306.

40. Di Stefano, A., Caramori, G., Barczyk, A., Vicari, C., Brun, P., Zanini, A., Cappello, F., Garofano, E., Padovani, A., Contoli, M. and Casolari, P.,

2014. Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD. *Thorax*, *69*(6), pp.516-524.

41. Dinarello, C., Arend, W., Sims, J., Smith, D., Blumberg, H., O'Neill, L., Goldbach-Mansky, R., Pizarro, T., Hoffman, H., Bufler, P. and Nold, M., 2010. IL-1 family nomenclature. *Nature immunology*, *11*(11), p.973.

42. Dinarello, C., Nold-Petry, C., Nold, M., Fujita, M., Li, S., Kim, S. and Bufler, P. (2016). Suppression of innate inflammation and immunity by interleukin-37. *European Journal of Immunology,* 46(5), pp.1067-1081.

43. Dinarello, C.A., 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology*, *27*, pp.519-550.

44. Dinarello, C.A., Nold-Petry, C., Nold, M., Fujita, M., Li, S., Kim, S. and Bufler, P., 2016. Suppression of innate inflammation and immunity by interleukin-37. *European journal of immunology*, *46*(5), pp.1067-1081.

45. Ding, C., Chen, X., Dascani, P., Hu, X., Bolli, R., Zhang, H., Mcleish, K. andYan, J. (2016). STAT3 Signaling in B Cells Is Critical for Germinal Center Maintenance and Contributes to the Pathogenesis of Murine Models of Lupus. *The Journal of Immunology*, 196(11), pp.4477-4486.

46. Ding, V.A., Zhu, Z., Xiao, H., Wakefield, M.R., Bai, Q. and Fang, Y., 2016. The role of IL-37 in cancer. *Medical oncology*, *33*(7), p.68.

47. Dullah, E.C. and Ongkudon, C.M., 2017. Current trends in endotoxin detection and analysis of endotoxin–protein interactions. *Critical reviews in biotechnology*, *37*(2), pp.251-261.

48. Eisenmesser, E.Z., Gottschlich, A., Redzic, J.S., Paukovich, N., Nix, J.C., Azam, T., Zhang, L., Zhao, R., Kieft, J.S., Meng, X. and Dinarello, C.A., 2019. Interleukin-37 monomer is the active form for reducing innate

immunity. *Proceedings of the National Academy of Sciences*, *116*(12), pp.5514-5522.

49. Ellisdon, A.M., Nold-Petry, C.A., D'Andrea, L., Cho, S.X., Lao, J.C., Rudloff, I., Ngo, D., Lo, C.Y., da Costa, T.P.S., Perugini, M.A. and Conroy, P.J., 2017. Homodimerization attenuates the anti-inflammatory activity of interleukin-37. *Science immunology*, *2*(8), p.eaaj1548.

50. Elmadani M, Khan S, Tenhunen O, Magga J, Aittokallio T, Wennerberg K, Kerkelä R. Novel Screening Method Identifies PI 3Kα, mTOR, and IGF 1R as Key Kinases Regulating Cardiomyocyte Survival. Journal of the American Heart Association. 2019 Nov 5;8(21):e013018.

51. Elmadani, M., Khan, S., Tenhunen, O., Magga, J., Aittokallio, T., Wennerberg, K. and Kerkelä, R., 2019. Novel Screening Method Identifies PI 3Kα, mTOR, and IGF 1R as Key Kinases Regulating Cardiomyocyte Survival. *Journal of the American Heart Association*, *8*(21), p.e013018.

52. Fàbrega, A. and Vila, J., 2013. Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. *Clinical microbiology reviews*, *26*(2), pp.308-341.

53. Fairweather, D., Frisancho-Kiss, S. and Rose, N.R., 2005. Viruses as adjuvants for autoimmunity: evidence from Coxsackievirus-induced myocarditis. *Reviews in medical virology*, *15*(1), pp.17-27.

54. Fierer, J. and Guiney, D.G., 2001. Diverse virulence traits underlying different clinical outcomes of Salmonella infection. *The Journal of clinical investigation*, *107*(7), pp.775-780.

55. Fonseca-Camarillo, G., Furuzawa-Carballeda, J. and Yamamoto-Furusho, J.K., 2015. Interleukin 35 (IL-35) and IL-37: Intestinal and peripheral

expression by T and B regulatory cells in patients with Inflammatory Bowel Disease. *Cytokine*, *75*(2), pp.389-402.

56. Garlanda, C., Anders, H. and Mantovani, A. (2009). TIR8/SIGIRR: an IL-1R/TLR family member with regulatory functions in inflammation and T cell polarization. *Trends in Immunology*, 30(9), pp.439-446.

57. Garlanda, C., Dinarello, C. and Mantovani, A. (2013). The Interleukin-1 Family: Back to the Future. *Immunity,* 39(6), pp.1003-1018.

58. Garlanda, C., Riva, F., Polentarutti, N., Buracchi, C., Sironi, M., De Bortoli, M., Muzio, M., Bergottini, R., Scanziani, E., Vecchi, A. and Hirsch, E., 2004. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. *Proceedings of the National Academy of Sciences*, *101*(10), pp.3522-3526.

59. Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J. and Madara, J.L., 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *The Journal of Immunology*, *167*(4), pp.1882-1885.

60. Godaly, G., Bergsten, G., Hang, L., Fischer, H., Frendeus, B., Lundstedt, A.C., Samuelsson, M., Samuelsson, P. and Svanborg, C., 2001. Neutrophil recruitment, chemokine receptors, and resistance to mucosal infection. *Journal of Leukocyte Biology*, *69*(6), pp.899-906.

61. Golubovskaya, V. and Wu, L., 2016. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. *Cancers*, *8*(3), p.36.

62. Gordon, S. M. et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* 36, 55–67 (2012)

63. Gowda, D.C., 2007. TLR-mediated cell signaling by malaria GPIs. *Trends in parasitology*, *23*(12), pp.596-604.

64. Grimsby, S., Jaensson, H., Dubrovska, A., Lomnytska, M., Hellman, U. and Souchelnytskyi, S. (2004). Proteomics-based identification of proteins interacting with Smad3: SREBP-2 forms a complex with Smad3 and inhibits its transcriptional activity. *FEBS Letters*, 577(1-2), pp.93-100.

65. Gu, J., Gao, X., Pan, X., Peng, X., Li, Y. and Li, M., 2015. High-level expression and one-step purification of a soluble recombinant human interleukin-37b in Escherichia coli. *Protein expression and purification*, *108*, pp.18-22.

66. Guillou, C., Derambure, C., Fréret, M., Verdet, M., Avenel, G., Golinski, M., Sabourin, J., Loarer, F., Adriouch, S., Boyer, O., Lequerré, T. and Vittecoq, O. (2015). Prophylactic Injection of Recombinant Alpha-Enolase Reduces Arthritis Severity in the Collagen-Induced Arthritis Mice Model. *PLOS ONE*, 10(8), p.e0136359.

67. Haabeth, O.A.W., Tveita, A.A., Fauskanger, M., Schjesvold, F., Lorvik, K.B., Hofgaard, P.O., Omholt, H., Munthe, L.A., Dembic, Z., Corthay, A. and Bogen, B., 2014. How do CD4+ T cells detect and eliminate tumor cells that either lack or express MHC class II molecules?. *Frontiers in immunology*, *5*, p.174.

68. Hanna, L.F., Matthews, T.D., Dinsdale, E.A., Hasty, D. and Edwards, R.A., 2012. Characterization of the ELPhiS prophage from Salmonella enterica serovar Enteritidis strain LK5. *Appl. Environ. Microbiol.*, *78*(6), pp.1785-1793.

69. Hardy, R. and Hayakawa, K. (2001). B Cell Development Pathways. *Annual Review of Immunology,* 19(1), pp. 595-621.

70. Hayakawa, H., Hayakawa, M., Kume, A. and Tominaga, S. (2007). Soluble ST2 Blocks Interleukin-33 Signaling in Allergic Airway Inflammation. *Journal of Biological Chemistry,* 282(36), pp.26369-26380.

71. Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Eugene, C.Y., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M. and Aderem, A., 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, *410*(6832), p.1099.

72. Healey, R., Lebhar, H., Hornung, S., Thordarson, P. and Marquis, C. (2017). An improved process for the production of highly purified recombinant thaumatin tagged-variants. *Food Chemistry*, 237, pp.825-832.

73. Højen, J.F., Rasmussen, T.A., Andersen, K.L.D., Winckelmann, A.A., Laursen, R.R., Gunst, J.D., Møller, H.J., Fujita, M., Østergaard, L., Søgaard, O.S. and Dinarello, C.A., 2015. Interleukin-37 expression is increased in chronic HIV-1-infected individuals and is associated with inflammation and the size of the total viral reservoir. *Molecular Medicine*, *21*(1), pp.337-345.

74. Holdsworth SR, Gan PY. Cytokines: names and numbers you should care about. Clinical Journal of the American Society of Nephrology. 2015 Dec 7;10(12):2243-54.

75. Hölscher C. The power of combinatorial immunology: IL-12 and IL-12-related dimeric cytokines in infectious diseases. Medical microbiology and immunology. 2004 Feb 1;193(1):1-7.

76. Huang, J., Hou, F., Zhang, A. and Li, Z. (2016). Protective effect of the polarity of macrophages regulated by IL-37 on atherosclerosis. *Genetics and Molecular Research,* 15(2).

77. Huang, Z., Gao, C., Chi, X., Hu, Y., Zheng, L., Zeng, T. and Wang, Q. (2015). IL-37 Expression is Upregulated in Patients with Tuberculosis and Induces Macrophages Towards an M2-like Phenotype. *Scandinavian Journal of Immunology,* 82(4), pp.370-379.

78. Huang, Z., Gao, C., Chi, X., Hu, Y.W., Zheng, L., Zeng, T. and Wang, Q., 2015. IL-37 expression is upregulated in patients with tuberculosis and induces macrophages towards an M2-like phenotype. *Scandinavian journal of immunology*, *82*(4), pp.370-379.

79. Imaeda, H., Takahashi, K., Fujimoto, T., Kasumi, E., Ban, H., Bamba, S., Sonoda, H., Shimizu, T., Fujiyama, Y. and Andoh, A. (2013). Epithelial expression of interleukin-37b in inflammatory bowel disease. *Clinical & Experimental Immunology,* 172(3), pp.410-416.

80. Ji, Q., Meng, K., Yu, K., Huang, S., Huang, Y., Min, X., Zhong, Y., Wu, B., Liu, Y., Nie, S. and Zhang, J., 2017. Exogenous interleukin 37 ameliorates atherosclerosis via inducing the Treg response in ApoE-deficient mice. *Scientific reports*, *7*(1), p.3310.

81. Jia, H., Liu, J. and Han, B. (2018). Reviews of Interleukin-37: Functions, Receptors, and Roles in Diseases. *BioMed Research International,* 2018, pp.1-14.

82. Jiang, X. and Chen, Z.J., 2012. The role of ubiquitylation in immune defence and pathogen evasion. *Nature Reviews Immunology*, *12*(1), p.35.

83. Jin, Y., Liu, L., Zhang, S., Tao, B., Tao, R., He, X., Qu, L., Huang, J., Wang, X. and Fu, Z., 2016. Chromium alters lipopolysaccharide-induced inflammatory responses both in vivo and in vitro. *Chemosphere*, *148*, pp.436-443.

84. Joseph, B.C., Pichaimuthu, S., Srimeenakshi, S., Murthy, M., Selvakumar, K., Ganesan, M. and Manjunath, S.R., 2015. An overview of the

parameters for recombinant protein expression in Escherichia coli. *J Cell Sci Ther*, *6*(05), p.1.

85. Jung, C., Meinzer, U., Montcuquet, N., Thachil, E., Château, D., Thiébaut, R., Roy, M., Alnabhani, Z., Berrebi, D., Dussaillant, M. and Pedruzzi, E., 2012. Yersinia pseudotuberculosis disrupts intestinal barrier integrity through hematopoietic TLR-2 signaling. *The Journal of clinical investigation*, *122*(6), pp.2239-2251.

86. Kalderon, D., Roberts, B.L., Richardson, W.D. and Smith, A.E., 1984. A short amino acid sequence able to specify nuclear location. *Cell*, *39*(3), pp.499-509.

87. Kalghatgi, S., Spina, C.S., Costello, J.C., Liesa, M., Morones-Ramirez, J.R., Slomovic, S., Molina, A., Shirihai, O.S. and Collins, J.J., 2013. Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in mammalian cells. *Science translational medicine*, *5*(192), pp.192ra85-192ra85.

88. Kanai, K., Satoh, Y., Yamanaka, H., Kawaguchi, A., Horie, K., Sugata, K., Hoshikawa, Y., Sata, T. and Sairenji, T., 2007. The vIL-10 gene of the Epstein-Barr virus (EBV) is conserved in a stable manner except for a few point mutations in various EBV isolates. *Virus genes*, *35*(3), pp.563-569.

89. Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. and Morishima, K., 2016. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic acids research*, *45*(D1), pp.D353-D361.

90. Kaplan, M.H., 2013. Th9 cells: differentiation and disease. *Immunological reviews*, 252(1), pp.104-115.

91. Kaur, J. and Reinhardt, D.P., 2012. Immobilized metal affinity chromatography co-purifies TGF-β1 with histidine-tagged recombinant extracellular proteins. *PLoS One*, *7*(10), p.e48629.

92. Kavathas PB, Boeras CM, Mulla MJ, Abrahams VM. Nod1, but not the ASC inflammasome, contributes to induction of IL-1β secretion in human trophoblasts after sensing of ChlaMyDia trachomatis. Mucosal immunology. 2013 Mar;6(2):235.

93. Keermann, M., Kõks, S., Reimann, E., Abram, K., Erm, T., Silm, H. and Kingo, K., 2015. Expression of IL-36 family cytokines and IL-37 but not IL-38 is altered in psoriatic skin. *Journal of dermatological science*, *80*(2), pp.150-152.

94. Kosmidis, C. and Denning, D.W., 2015. The clinical spectrum of pulmonary aspergillosis. *Thorax*, *70*(3), pp.270-277.

95. Koyama, F., Sawada, H., Fujii, H., Hirao, T., Ueno, M., Hamada, H. and Nakano, H., 2000. Enzyme/prodrug gene therapy for human colon cancer cells using adenovirus-mediated transfer of the Escherichia coli cytosine deaminase gene driven by a CAG promoter associated with 5-fluorocytosine administration. *Journal of experimental & clinical cancer research: CR*, *19*(1), pp.75-80.

96. Kumar, S., Hanning, C.R., Brigham-Burke, M.R., Rieman, D.J., Lehr, R., Khandekar, S., Kirkpatrick, R.B., Scott, G.F., Lee, J.C., Lynch, F.J. and Gao, W., 2002. Interleukin-1F7B (IL-1H4/IL-1F7) is processed by caspase-1 and mature IL-1F7B binds to the IL-18 receptor but does not induce IFN-γ production. *Cytokine*, *18*(2), pp.61-71.

97. Lassoued, K. (1993). Expression of surrogate light chain receptors is restricted to a late stage in pre-B cell differentiation. *Cell,* 73(1), pp.73-86.

98. Lech, M., Garlanda, C., Mantovani, A., Kirschning, C., Schlöndorff, D. and Anders, H. (2007). Different roles of TiR8/Sigirr on toll-like receptor signaling in intrarenal antigen-presenting cells and tubular epithelial cells. *Kidney International*, 72(2), pp.182-192.

99. Léger B, Cartoni R, Praz M, Lamon S, Dériaz O, Crettenand A, Gobelet C, Rohmer P, Konzelmann M, Luthi F, Russell AP. Akt signalling through GSK-3β, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. The Journal of physiology. 2006 Nov 1;576(3):923-33.

100. Leveque, G., Forgetta, V., Morroll, S., Smith, A.L., Bumstead, N., Barrow, P., Loredo-Osti, J.C., Morgan, K. and Malo, D., 2003. Allelic variation in TLR4 is linked to susceptibility to Salmonella enterica serovar Typhimurium infection in chickens. *Infection and immunity*, *71*(3), pp.1116-1124.

101. Levings, M.K. and Schrader, J.W., 1999. IL-4 inhibits the production of TNF-α and IL-12 by STAT6-dependent and-independent mechanisms. *The Journal of Immunology*, *162*(9), pp.5224-5229.

102. Li, D., Zhang, X. and Chen, B., 2015. SIGIRR participates in negative regulation of LPS response and tolerance in human bladder epithelial cells. *BMC immunology*, *16*(1), p.73.

103. Li, J., Zhai, Y., Ao, L., Hui, H., Fullerton, D., Dinarello, C. and Meng, X. (2017). Interleukin-37 suppresses the inflammatory response to protect cardiac function in old endotoxemic mice. *Cytokine*, 95, pp.55-63.

104. Li, S., Neff, C.P., Barber, K., Hong, J., Luo, Y., Azam, T., Palmer, B.E., Fujita, M., Garlanda, C., Mantovani, A. and Kim, S., 2015. Extracellular forms of IL-37 inhibit innate inflammation in vitro and in vivo but require the IL-1 family decoy receptor IL-1R8. *Proceedings of the National Academy of Sciences*, *112*(8), pp.2497-2502.

105. Li, Y., Wang, Z., Yu, T., Chen, B., Zhang, J., Huang, K. and Huang, Z., 2014. Increased expression of IL-37 in patients with Graves' disease and its contribution to suppression of proinflammatory cytokines production in peripheral blood mononuclear cells. *PloS one*, *9*(9), p.e107183.

106. Li, Z., Zhang, C., Zhou, Z., Zhang, J., Zhang, J. and Tian, Z., 2012. Small intestinal intraepithelial lymphocytes expressing CD8 and T cell receptor γδ are involved in bacterial clearance during Salmonella enterica serovar Typhimurium infection. *Infection and immunity*, *80*(2), pp.565-574.

107. Liew, F.Y., Xu, D., Brint, E.K. and O'Neill, L.A., 2005. Negative regulation of toll-like receptor-mediated immune responses. *Nature Reviews Immunology*, *5*(6), p.446.

108. Liu H, Zheng R, Wang P, Yang H, He X, Ji Q, Bai W, Chen H, Chen J, Peng W, Liu S. IL-37 confers protection against mycobacterial infection involving suppressing inflammation and modulating T cell activation. PloS one. 2017 Jan 11;12(1):e0169922.

109. Liu, J., Lin, J., He, S., Wu, C., Wang, B., Liu, J., Duan, Y., Liu, T., Shan, S., Yang, K., Dong, N., Ji, Q., Huang, K. and Li, D. (2018). Transgenic Overexpression of IL-37 Protects Against Atherosclerosis and Strengthens Plaque Stability. *Cellular Physiology and Biochemistry*, 45(3), pp.1034-1050.

110. Liu, L., Xue, Y., Zhu, Y., Xuan, D., Yang, X., Liang, M., Wang, J., Zhu, X., Zhang, J. and Zou, H., 2016. Interleukin 37 limits monosodium urate crystal-induced innate immune responses in human and murine models of gout. *Arthritis research & therapy*, *18*(1), p.268.

111. Liu, Y., Zhang, J., Lane, P., Chan, E. and Maclennan, I. (1991). Sites of specific B cell activation in primary and secondary responses to T cell-

dependent and T cell-independent antigens. *European Journal of Immunology,* 21(12), pp. 2951-2962.

112. Lo, W.L., Shah, N.H., Ahsan, N., Horkova, V., Stepanek, O., Salomon, A.R., Kuriyan, J. and Weiss, A., 2018. Lck promotes Zap70-dependent LAT phosphorylation by bridging Zap70 to LAT. *Nature immunology*, *19*(7), p.733.

113. Long, S. and Buckner, J. (2011). CD4+FOXP3+ T Regulatory Cells in Human Autoimmunity: More Than a Numbers Game. *The Journal of Immunology,* 187(5), pp.2061-2066.

114. Lunding, L., Webering, S., Vock, C., Schröder, A., Raedler, D., Schaub, B., Fehrenbach, H. and Wegmann, M., 2015. IL-37 requires IL-18Rα and SIGIRR/IL-1R8 to diminish allergic airway inflammation in mice. *Allergy*, *70*(4), pp.366-373.

115. Luo H, Hao E, Tan D, Wei W, Xie J, Feng X, Du Z, Huang C, Bai G, Hou Y, Cheng C. Apoptosis effect of Aegiceras corniculatum on human colorectal cancer via activation of FoxO signaling pathway. Food and Chemical Toxicology. 2019 Oct 1:110861.

116. Luo, Y., Cai, X., Liu, S., Wang, S., Nold-Petry, C.A., Nold, M.F., Bufler, P., Norris, D., Dinarello, C.A. and Fujita, M., 2014. Suppression of antigen-specific adaptive immunity by IL-37 via induction of tolerogenic dendritic cells. *Proceedings of the National Academy of Sciences*, *111*(42), pp.15178-15183.

117. Lynn, D., Chan, C., Naseer, M., Yau, M., Lo, R., Sribnaia, A., Ring, G., Que, J., Wee, K., Winsor, G., Laird, M., Breuer, K., Foroushani, A., Brinkman, F. and Hancock, R. (2010). Curating the innate immunity interactome. *BMC Systems Biology,* 4(1), p.117.

118. Macedo, B. and Cordeiro, Y., 2017. Unraveling prion protein interactions with aptamers and other PrP-binding nucleic acids. *International journal of molecular sciences*, *18*(5), p.1023.

119. MALAEB, M., BIZRI, A., GHOSN, N., BERRY, A. and MUSHARRAFIEH, U. (2016). Salmonella burden in Lebanon. *Epidemiol. Infect.*, pp.1-9.

120. Marshall, G. (1992). Cytokines: Clinical Potentials for the Allergic Patient. *Allergy and Asthma Proceedings,* 13(6), pp.311-315.

121. Martinez, F.O. and Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports*, *6*.

122. McCurdy, S., Baumer, Y., Toulmin, E., Lee, B.H. and Boisvert, W.A., 2017. Macrophage-specific expression of IL-37 in hyperlipidemic mice attenuates atherosclerosis. *The Journal of Immunology*, *199*(10), pp.3604-3613.

123. McNamee, E., Masterson, J., Jedlicka, P., McManus, M., Grenz, A., Collins, C., Nold, M., Nold-Petry, C., Bufler, P., Dinarello, C. and Rivera-Nieves, J. (2011). Interleukin 37 expression protects mice from colitis. *Proceedings of the National Academy of Sciences,* 108(40), pp.16711-16716.

124. McNamee, E.N., Masterson, J.C., Jedlicka, P., McManus, M., Grenz, A., Collins, C.B., Nold, M.F., Nold-Petry, C., Bufler, P., Dinarello, C.A. and Rivera-Nieves, J., 2011. Interleukin 37 expression protects mice from colitis. *Proceedings of the National Academy of Sciences*, *108*(40), pp.16711-16716.

125. Medzhitov R. (2001). Toll-like receptors and innate immunity. *Nat Rev Immunol*, 1(2), PP.135–145.

126. Meffre, E. and Wardemann, H. (2008). B-cell tolerance checkpoints in health and autoimmunity. *Current Opinion in Immunology,* 20(6), pp.632-638.

127. Mittrücker, H.W. and Kaufmann, S.H., 2000. Immune response to infection with Salmonella typhimurium in mice. *Journal of leukocyte biology*, *67*(4), pp.457-463.

128. Mittrücker, H.W., Köhler, A. and Kaufmann, S.H., 2002. Characterization of the murine T-lymphocyte response to Salmonella enterica serovar Typhimurium infection. *Infection and immunity*, *70*(1), pp.199-203.

129. Mølvig, J. and Baek, L., 1987. Removal of Endotoxin from Culture Media by a Polymyxin B Sepharose Column: The Activity of Contaminating Endotoxin in Culture Media Measured by the Interleukin 1 Inducing Effect on Human Monocyte Cultures and by the Limudus Test. *Scandinavian journal of immunology*, *26*(6), pp.611-619.

130. Moreira L, Zamboni DS. NOD1 and NOD2 signaling in infection and inflammation. Frontiers in immunology. 2012 Nov 8;3:328.

131. Moretti S, Bozza S, Oikonomou V, Renga G, Casagrande A, Iannitti RG, Puccetti M, Garlanda C, Kim S, Li S, van de Veerdonk FL. IL-37 inhibits inflammasome activation and disease severity in murine aspergillosis. PLoS pathogens. 2014 Nov 6;10(11):e1004462.

132. Moretti, S., Bozza, S., Oikonomou, V., Renga, G., Casagrande, A., Iannitti, R.G., Puccetti, M., Garlanda, C., Kim, S., Li, S. and van de Veerdonk, F.L., 2014. IL-37 inhibits inflammasome activation and disease severity in murine aspergillosis. *PLoS pathogens*, *10*(11), p.e1004462.

133. Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J., Ohtani, M., Fujii, H. and Koyasu, S. (2009). Innate

production of TH2 cytokines by adipose tissue-associated c-Kit+Sca-1+ lymphoid cells. *Nature,* 463(7280), pp.540-544.

134. Moss, R.B., Moll, T., El-Kalay, M., Kohne, C., Soo Hoo, W., Encinas, J. and Carlo, D.J., 2004. Th1/Th2 cells in inflammatory disease states: therapeutic implications. *Expert opinion on biological therapy*, *4*(12), pp.1887-1896.

135. Mungrue, I.N., Husain, M. and Stewart, D.J., 2004. The role of NOS in heart failure: lessons from murine genetic models. In *The Role of Nitric Oxide in Heart Failure* (pp. 113-128). Springer, Boston, MA.

136. Murphy, K. and Weaver, C., 2016. *Janeway's immunobiology*. Garland Science.

137. Murphy, R. and Roberts, C. (2013). Protein misfolding and aggregation research: Some thoughts on improving quality and utility. *Biotechnology Progress,* 29(5), pp.1109-1115.

138. Nagalakshmi, U., Waern, K. and Snyder, M., 2010. RNA-Seq: a method for comprehensive transcriptome analysis. *Current protocols in molecular biology*, *89*(1), pp.4-11.

139. Nallamsetty, S., Austin, B.P., Penrose, K.J. and Waugh, D.S., 2005. Gateway vectors for the production of combinatorially-tagged His6-MBP fusion proteins in the cytoplasm and periplasm of Escherichia coli. *Protein Science*, *14*(12), pp.2964-2971.

140. Namen, A., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D., Schmierer, A., Mosley, B., March, C., Urdal, D., Gillis, S., Cosman, D. and Goodwin, R. (1988). Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature,* 333(6173), pp.571-573.

141. Nicklin, M.J., Barton, J.L., Nguyen, M., FitzGerald, M.G., Duff, G.W. and Kornman, K., 2002. A sequence-based map of the nine genes of the human interleukin-1 cluster. *Genomics*, *79*(5), pp.718-725.

142. Nishimoto, N., Kubagawa, H., Ohno, T., Gartland, G., Stankovic, A. and Cooper, M. (1991). Normal pre-B cells express a receptor complex of mu heavy chains and surrogate light-chain proteins. *Proceedings of the National Academy of Sciences,* 88(14), pp.6284-6288.

143. Nold, M., Nold-Petry, C., Zepp, J., Bufler, P. and Dinarello, C. (2009). IL-1 family member 7 is a fundamental inhibitor of innate immunity. *Cytokine,* 48(1-2), pp.68-69.

144. Nold, M., Nold-Petry, C., Zepp, J., Palmer, B., Bufler, P. and Dinarello, C. (2010). IL-37 is a fundamental inhibitor of innate immunity. *Nature Immunology,* 11(11), pp.1014-1022.

145. Nold, M.F., Nold-Petry, C.A., Lo, C., Li, S., Rudloff, I., Zepp, J.A., Azam, T., Bufler, P., Garlanda, C., Mantovani, A. and Dinarello, C.A., 2013. 187: interleukin 37 employs the IL-1 family inhibitory receptor SIGIRR and the alpha chain of the IL-18 receptor to suppress innate immunity. *Cytokine*, *63*(3), p.287.

146. Nold-Petry, C.A., Lo, C.Y., Rudloff, I., Elgass, K.D., Li, S., Gantier, M.P., Lotz-Havla, A.S., Gersting, S.W., Cho, S.X., Lao, J.C. and Ellisdon, A.M., 2015. IL-37 requires the receptors IL-18Rα and IL-1R8 (SIGIRR) to carry out its multifaceted anti-inflammatory program upon innate signal transduction. *Nature immunology*, *16*(4), p.354.

147. Onwuezobe, I.A., Oshun, P.O. and Odigwe, C.C., 2012. Antimicrobials for treating symptomatic non-typhoidal Salmonella infection. *Cochrane Database of Systematic Reviews*, (11).

148. O'Shea, J. and Paul, W. (2010). Mechanisms Underlying Lineage Commitment and Plasticity of Helper CD4+ T Cells. *Science,* 327(5969), pp. 1098-1102.

149. Pan JX. LncRNA H19 promotes atherosclerosis by regulating MAPK and NF-kB signaling pathway. Eur Rev Med Pharmacol Sci. 2017 Jan 1;21(2):322-8.

150. Pan, G., Risser, P., Mao, W., Baldwin, D.T., Zhong, A.W., Filvaroff, E., Yansura, D., Lewis, L., Eigenbrot, C., Henzel, W.J. and Vandlen, R., 2001. IL-1H, an interleukin 1-related protein that binds IL-18 receptor/IL-1Rrp. *Cytokine*, *13*(1), pp.1-7.

151. Parisi, L., Gini, E., Baci, D., Tremolati, M., Fanuli, M., Bassani, B., Farronato, G., Bruno, A. and Mortara, L., 2018. Macrophage polarization in chronic inflammatory diseases: Killers or builders?. *Journal of immunology research*, *2018*.

152. Pashine, A., John, B., Rath, S., George, A. and Bal, V., 1999. Th1 dominance in the immune response to live Salmonella typhimurium requires bacterial invasiveness but not persistence. *International immunology*, *11*(4), pp.481-489.

153. Paterson, G.K. and Orihuela, C.J., 2010. Pneumococci: immunology of the innate host response. *Respirology*, *15*(7), pp.1057-1063.

154. Patton, J.B., Bonne-Année, S., Deckman, J., Hess, J.A., Torigian, A., Nolan, T.J., Wang, Z., Kliewer, S.A., Durham, A.C., Lee, J.J. and Eberhard, M.L., 2018. Methylprednisolone acetate induces, and Δ7-dafachronic acid suppresses, Strongyloides stercoralis hyperinfection in NSG mice. *Proceedings of the National Academy of Sciences*, *115*(1), pp.204-209.

155. Percival, S., Yates, M., Williams, D., Chalmers, R. and Gray, N. (n.d.). Microbiology of waterborne diseases.Fabrega, A. and Vila, J.

(2013). Salmonella enterica Serovar Typhimurium Skills To Succeed in the Host: Virulence and Regulation. *Clinical Microbiology Reviews*, 26(2), pp.308-341.

156. Percival, S.L., Chalmers, R., Embrey, M., Hunter, P.R., Sellwood, J. and Wyn-Jones, P., 2004. Microbiology of waterborne diseases.

157. Pesarrodona, M., Unzueta, U. and Vázquez, E., 2015. Dialysis: A characterization method of aggregation tendency. In *Insoluble Proteins* (pp. 321-330). Humana Press, New York, NY.

158. Plantinga, M., Hammad, H. and Lambrecht, B. (2010). Origin and functional specializations of DC subsets in the lung. *European Journal of Immunology,* 40(8), pp.2112-2118.

159. Qin, J., Qian, Y., Yao, J., Grace, C. & Li, X. (2005). SIGIRR inhibits interleukin-1 receptorand toll-like receptor 4-mediated signaling through different mechanisms. J. Biol. *Chem.* 280, 25233–25241.

160. Quirk, S. and Agrawal, D. (2014). Immunobiology of IL-37: mechanism of action and clinical perspectives. *Expert Review of Clinical Immunology,* 10(12), pp.1703-1709.

161. Raphael, I., Nalawade, S., Eagar, T.N. and Forsthuber, T.G., 2015. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine*, *74*(1), pp.5-17.

162. Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R. and Schwartz, M. (2007). Toll-like receptors modulate adult hippocampal neurogenesis. *Nature Cell Biology*, 9(9), pp.1081-1088.

163. Rosano, G.L. and Ceccarelli, E.A., 2014. Recombinant protein expression in Escherichia coli: advances and challenges. *Frontiers in microbiology*, *5*, p.172.

164. Roussel, L., Farias, R. and Rousseau, S., 2013. IL-33 is expressed in epithelia from patients with cystic fibrosis and potentiates neutrophil recruitment. *Journal of Allergy and Clinical Immunology*, *131*(3), pp.913-916.

165. Rudloff, I., Cho, S., Lao, J., Ngo, D., McKenzie, M., Nold-Petry, C. and Nold, M. (2016). Monocytes and dendritic cells are the primary sources of interleukin 37 in human immune cells. *Journal of Leukocyte Biology,* 101(4), pp.901-911.

166. Sabbagh, S.C., Forest, C.G., Lepage, C., Leclerc, J.M. and Daigle, F., 2010. So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi. *FEMS microbiology letters*, *305*(1), pp.1-13.

167. Sattler, S., Smits, H., Xu, D. and Huang, F. (2013). The Evolutionary Role of the IL-33/ST2 System in Host Immune Defence. *Archivum Immunologiae et Therapiae Experimentalis,* 61(2), pp. 107-117.

168. Scallan, E., Hoekstra, R., Angulo, F., Tauxe, R., Widdowson, M., Roy, S., Jones, J. and Griffin, P. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerg. Infect. Dis.,* 17(1), pp.7-15.

169. Schauer, A.E., Klassert, T.E., von Lachner, C., Riebold, D., Schneeweiß, A., Stock, M., Müller, M.M., Hammerschmidt, S., Bufler, P., Seifert, U. and Dietert, K., 2017. IL-37 causes excessive inflammation and tissue damage in murine pneumococcal pneumonia. *Journal of innate immunity*, *9*(4), pp.403-418.

170. Schlissel, M. and Baltimore, D. (1989). Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. *Cell,* 58(5), pp.1001-1007.

171. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005 Nov 1;23(5):479-90.

172. Sharma, S., Kulk, N., Nold, M.F., Gräf, R., Kim, S.H., Reinhardt, D., Dinarello, C.A. and Bufler, P., 2008. The IL-1 family member 7b translocates to the nucleus and down-regulates proinflammatory cytokines. *The Journal of Immunology*, *180*(8), pp.5477-5482.

173. Shuai, X., Wei-Min, L., Tong, Y.L., Dong, N., Sheng, Z.Y. and Yao, Y.M., 2015. Expression of IL-37 contributes to the immunosuppressive property of human CD4+ CD25+ regulatory T cells. *Scientific reports*, *5*, p.14478.

174. Singh, A., Upadhyay, V., Upadhyay, A.K., Singh, S.M. and Panda, A.K., 2015. Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. *Microbial cell factories*, *14*(1), p.41.

175. Smithrithee, R., Niyonsaba, F., Kiatsurayanon, C., Ushio, H., Ikeda, S., Okumura, K. and Ogawa, H., 2015. Human β-defensin-3 increases the expression of interleukin-37 through CCR6 in human keratinocytes. *Journal of dermatological science*, *77*(1), pp.46-53.

176. Song, L., Qiu, F., Fan, Y., Ding, F., Liu, H., Shu, Q., Liu, W. and Li, X., 2013. Glucocorticoid regulates interleukin-37 in systemic lupus erythematosus. *Journal of Clinical Immunology*, *33*(1), pp.111-117.

177. Sørensen, H.P. and Mortensen, K.K., 2005. Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli. *Microbial cell factories*, *4*(1), p.1.

178. Southcombe, J.H., Redman, C.W.G., Sargent, I.L. and Granne, I., 2015. Interleukin-1 family cytokines and their regulatory proteins in normal pregnancy and pre-eclampsia. *Clinical & Experimental Immunology*, *181*(3), pp.480-490.

179. Spartalis, M., Tzatzaki, E., Spartalis, E., Damaskos, C., Mavrogeni, S. and Voudris, V., 2017. Parvovirus B19 myocarditis of fulminant evolution. *Cardiology research*, *8*(4), p.172.

180. Spears, M., McSharry, C., Donnelly, I., Jolly, L., Brannigan, M., Thomson, J., Lafferty, J., Chaudhuri, R., Shepherd, M., Cameron, E. and Thomson, N. (2011).Peripheral blood dendritic cell subtypes are significantly elevated in subjects with asthma. *Clinical & Experimental Allergy*, 41(5), pp.665-672.

181. Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J., Eberl, G., Koyasu, S., Locksley, R., McKenzie, A., Mebius, R., Powrie, F. and Vivier, E. (2013). Innate lymphoid cells — a proposal for uniform nomenclature. *Nature Reviews Immunology*, 13(2), pp.145-149.

182. Stuehr, D.J., Cho, H.J., Kwon, N.S., Weise, M.F. and Nathan, C.F., 1991. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD-and FMN-containing flavoprotein. *Proceedings of the National Academy of Sciences*, 88(17), pp.7773-7777.

183. Tang, D., Kang, R., Coyne, C.B., Zeh, H.J. and Lotze, M.T., 2012. PAMP s and DAMP s: signal 0s that spur autophagy and immunity. *Immunological reviews*, 249(1), pp.158-175.

184. Takeuchi, O., Kawai, T., Sanjo, H., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Takeda, K. and Akira, S., 1999. TLR6: A novel member of an expanding toll-like receptor family. *Gene*, *231*(1-2), pp.59-65.

185. Tardieu, M., Zérah, M., Husson, B., de Bournonville, S., Deiva, K., Adamsbaum, C., Vincent, F., Hocquemiller, M., Broissand, C., Furlan, V. and Ballabio, A., 2014. Intracerebral administration of adeno-associated viral vector serotype rh. 10 carrying human SGSH and SUMF1 cDNAs in children with mucopolysaccharidosis type IIIA disease: results of a phase I/II trial. *Human gene therapy*, *25*(6), pp.506-516.

186. Tawfik, M.G., Nasef, S.I., Omar, H.H. and Ghaly, M.S., 2017. Serum Interleukin-37: a new player in Lupus Nephritis?. *International journal of rheumatic diseases*, *20*(8), pp.996-1001.

187. Taylor, S.L., Renshaw, B.R., Garka, K.E., Smith, D.E. and Sims, J.E., 2002. Genomic organization of the interleukin-1 locus. *Genomics*, *79*(5), pp.726-733.

188. Teng, X., Hu, Z., Wei, X., Wang, Z., Guan, T., Liu, N., Liu, X., Ye, N., Deng, G., Luo, C. and Huang, N., 2014. IL-37 ameliorates the inflammatory process in psoriasis by suppressing proinflammatory cytokine production. *The Journal of Immunology*, *192*(4), pp.1815-1823.

189. Thomson, A. and Lotze, M. (2003). The cytokine handbook. *Amsterdam: Academic Press*.

190. Toshchakov, V., Jones, B., Perera, P., Thomas, K., Cody, M., Zhang, S., Williams, B., Major, J., Hamilton, T., Fenton, M. and Vogel, S. (2002). TLR4, but not TLR2, mediates IFN-ß–induced STAT1α/ß-dependent gene expression in macrophages. *Nature Immunology*, 3(4), pp.392-398.

191. Trebichavský, I., Šplíchal, I. and Šplíchalová, A. (2010). Innate immune response in the gut against Salmonella — review. Folia Microbiol, 55(3), pp.295-300.

192. Trieu, A., Bokil, N., Dunn, J.A., Roberts, T.L., Xu, D., Liew, F.Y., Hume, D.A., Stacey, K.J. and Sweet, M.J., 2009. TLR9-independent effects of inhibitory oligonucleotides on macrophage responses to S. typhimurium. *Immunology and cell biology*, *87*(3), pp.218-225.

193. Tsolis, R.M., Young, G.M., Solnick, J.V. and Bäumler, A.J., 2008. From bench to bedside: stealth of enteroinvasive pathogens. *Nature Reviews Microbiology*, *6*(12), p.883.

194. Tükel, Ç., Raffatellu, M., Humphries, A.D., Wilson, R.P., Andrews-Polymenis, H.L., Gull, T., Figueiredo, J.F., Wong, M.H., Michelsen, K.S., Akçelik, M. and Adams, L.G., 2005. CsgA is a pathogen-associated molecular pattern of Salmonella enterica serotype Typhimurium that is recognized by Toll-like receptor 2. *Molecular microbiology*, *58*(1), pp.289-304.

195. van de Veerdonk, F.L., Gresnigt, M.S., Oosting, M., van der Meer, J.W., Joosten, L.A., Netea, M.G. and Dinarello, C.A., 2015. Protective host defense against disseminated candidiasis is impaired in mice expressing human interleukin-37. *Frontiers in microbiology*, *5*, p.762.

196. Vazquez-Torres, A., Stevanin, T., Jones-Carson, J., Castor, M., Read, R. C., & Fang, F. C. (2008). Analysis of Nitric Oxide-Dependent Antimicrobial Actions in Macrophages and Mice. *Methods in enzymology*, 437, 521-538.

197. Vazquez-Torres, A., Stevanin, T., Jones-Carson, J., Castor, M., Read, R.C. and Fang, F.C., 2008. Analysis of nitric oxide-dependent antimicrobial actions in macrophages and mice. In *Methods in enzymology* (Vol. 437, pp. 521-538). Academic Press.

198. Veerdonk, F.V.D. and Netea, M., 2013. New insights in the immunobiology of IL-1 family members. *Frontiers in immunology*, *4*, p.167.

199. Vlahopoulos S, Boldogh I, Casola A, Brasier AR. Nuclear Factor-κB–Dependent Induction of Interleukin-8 Gene Expression by Tumor Necrosis Factor□: Evidence for an Antioxidant Sensitive Activating Pathway Distinct From Nuclear Translocation. Blood. 1999 Sep 15;94(6):1878-89.

200. Wald, D., Qin, J., Zhao, Z., Qian, Y., Naramura, M., Tian, L., Towne, J., Sims, J.E., Stark, G.R. and Li, X., 2003. SIGIRR, a negative regulator of Toll-like receptor–interleukin 1 receptor signaling. *Nature immunology*, *4*(9), p.920.

201. Walker, J., Barlow, J. and McKenzie, A. (2013). Innate lymphoid cells — how did we miss them?. *Nature Reviews Immunology,* 13(2), pp.75-87.

202. Walls, D. and Loughran, S.T., 2011. Tagging recombinant proteins to enhance solubility and aid purification. In *Protein Chromatography* (pp. 151-175). Humana Press.

203. Wang X, Zhan E, Lu G, Mu Q, Zhang T, Yang N. Sphingosine-1-Phosphate Improves the Biological Features of Mouse Bone Marrow-Derived EPCs Partially through PI3K/AKT/eNOS/NO Pathway. Molecules. 2019 Jan;24(13):2404.

204. Wang, C., Deng, L., Hong, M. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature, 412(6844), PP. 346–351. 34- Takeuchi, O., Takeda, K., Hoshino, K., Adachi, O., Ogawa, T. and Akira, S. (2000). Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *International Immunology,* 12(1), pp.113-117.

205. Wang, L., Quan, Y., Yue, Y., Heng, X. and Che, F., 2018. Interleukin-37: A crucial cytokine with multiple roles in disease and potentially clinical therapy. *Oncology letters*, *15*(4), pp.4711-4719.

206. Wang, S., An, W., Yao, Y., Chen, R., Zheng, X., Yang, W., Zhao, Y., Hu, X., Jiang, E., Bie, Y. and Chen, Z., 2015. Interleukin 37 expression inhibits STAT3 to suppress the proliferation and invasion of human cervical cancer cells. *Journal of Cancer*, *6*(10), p.962.

207. Wang, X., Xu, K., Chen, S., Li, Y. and Li, M., 2018. Role of interleukin-37 in inflammatory and autoimmune diseases. *Iranian Journal of Immunology*, *15*(3), pp.165-174.

208. Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L. and Lepore, R., 2018. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic acids research*, *46*(W1), pp.W296-W303.

209. Weaver, C., Elson, C., Fouser, L. and Kolls, J. (2013). The Th17 Pathway and Inflammatory Diseases of the Intestines, Lungs, and Skin. *Annual Review of Pathology: Mechanisms of Disease,* 8(1), pp.477-512.

210. Xia, T., Zheng, X.F., Qian, B.H., Fang, H., Wang, J.J., Zhang, L.L., Pang, Y.F., Zhang, J., Wei, X.Q., Xia, Z.F. and Zhao, D.B., 2015. Plasma interleukin-37 is elevated in patients with rheumatoid arthritis: its correlation with disease activity and Th1/Th2/Th17-related cytokines. *Disease markers*, *2015*.

211. Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J. and Zhang, Y., 2015. The I-TASSER Suite: protein structure and function prediction. *Nature methods*, *12*(1), p.7.

212. Yang, Z., Zhang, L., Zhang, Y., Zhang, T., Feng, Y., Lu, X., Lan, W., Wang, J., Wu, H., Cao, C. and Wang, X., 2011. Highly efficient production

of soluble proteins from insoluble inclusion bodies by a two-step-denaturing and refolding method. *PloS one*, *6*(7), p.e22981.

213. Ye, L., Ji, L., Wen, Z., Zhou, Y., Hu, D., Li, Y., Yu, T., Chen, B., Zhang, J., Ding, L. and Du, J., 2014. IL-37 inhibits the production of inflammatory cytokines in peripheral blood mononuclear cells of patients with systemic lupus erythematosus: its correlation with disease activity. *Journal of translational medicine*, *12*(1), p.69.

214. Ye, Z., Wang, C., Kijlstra, A., Zhou, X. and Yang, P., 2014. A possible role for interleukin 37 in the pathogenesis of Behcet's disease. *Current molecular medicine*, *14*(4), pp.535-542.

215. Yurasov, S., Wardemann, H., Hammersen, J., Tsuiji, M., Meffre, E., Pascual, V. and Nussenzweig, M. (2005). Defective B cell tolerance checkpoints in systemic lupus erythematosus. *The Journal of Experimental Medicine,* 201(5), pp.703-711.

216. Zhan, Q. and Zeng, Q. (2017). IL-37 Suppresses MyD88-mediated Inflammatory Responses in Human Aortic Valve Interstitial Cells. *Molecular Medicine,* 23(1), p.1.

217. Zhan, R., Han, Q., Zhang, C., Tian, Z. and Zhang, J., 2015. Toll-like receptor 2 (TLR2) and TLR9 play opposing roles in host innate immunity against Salmonella enterica serovar Typhimurium infection. *Infection and immunity*, *83*(4), pp.1641-1649.

218. Zhang, J., Yang, J., Xu, X., Liang, L., Sun, H., Liu, G., Zhang, L. and Su, Y., 2016. The influence of genetic polymorphisms in TLR4 and TIRAP, and their expression levels in peripheral blood, on susceptibility to sepsis. *Experimental and therapeutic medicine*, *11*(1), pp.131-139.

219. Zhang, J.A., Liu, G.B., Zheng, B.Y., Lu, Y.B., Gao, Y.C., Cai, X.Z., Dai, Y.C., Yu, S.Y., Jia, Y., Chen, C. and Zhuang, Z.G., 2016. Tuberculosis-

sensitized monocytes sustain immune response of interleukin-37. *Molecular immunology*, *79*, pp.14-21.

220. Zhang, L., Pan, Z., Kang, X., Yang, Y., Kang, H., Zhang, N., Rosati, J.M. and Jiao, X., 2015. Amino acids 89–96 of Salmonella typhimurium flagellin represent the major domain responsible for TLR5-independent adjuvanticity in the humoral immune response. *Cellular & molecular immunology*, *12*(5), p.625.

221. Zhang, Y., Lu, Y., Ma, L., Cao, X., Xiao, J., Chen, J., Jiao, S., Gao, Y., Liu, C., Duan, Z. and Li, D., 2014. Activation of vascular endothelial growth factor receptor-3 in macrophages restrains TLR4-NF-κB signaling and protects against endotoxin shock. *Immunity*, *40*(4), pp.501-514.

222. Zhao X, Kwan JY, Yip K, Liu PP, Liu FF. Targeting metabolic dysregulation for fibrosis therapy. Nature Reviews Drug Discovery. 2019 Sep 23:1-9.

223. Zhao, M., Hu, Y., Shou, J., Su, S.B., Yang, J. and Yang, T., 2017. IL-37 impairs host resistance to listeria infection by suppressing macrophage function. *Biochemical and biophysical research communications*, *485*(2), pp.563-568.

224. Zhao, M., Li, Y., Guo, C., Wang, L., Chu, H., Zhu, F., Li, Y., Wang, X., Wang, Q., Zhao, W. and Shi, Y., 2018. IL-37 isoform D downregulates pro-inflammatory cytokines expression in a Smad3-dependent manner. *Cell death & disease*, *9*(6), p.582.

225. Zhao, M., Li, Y., Guo, C., Wang, L., Chu, H., Zhu, F., Li, Y., Wang, X., Wang, Q., Zhao, W., Shi, Y., Chen, W. and Zhang, L. (2018). IL-37 isoform D downregulates pro-inflammatory cytokines expression in a Smad3-dependent manner. *Cell Death & Disease,* 9(6).

226. Zhao, P.W., Jiang, W.G., Wang, L., Jiang, Z.Y., Shan, Y.X. and Jiang, Y.F., 2014. Plasma levels of IL-37 and correlation with TNF-α, IL-17A, and

disease activity during DMARD treatment of rheumatoid arthritis. *PloS one*, *9*(5), p.e95346.

227. Zhao, S., Zhang, B., Zhang, Y., Gordon, W., Du, S., Paradis, T., Vincent, M. and von Schack, D., 2016. Bioinformatics for RNA-Seq Data Analysis. *Bioinformatics—Updated Features and Applications: InTech*, pp.125-49.

228. Zhou, F., Zhu, C.L., Niu, Z.L., Xu, F.X., Song, H. and Liu, X.H., 2019. Influenza A virus inhibits influenza virus replication by inducing IL-37. *Journal of clinical laboratory analysis*, *33*(1), p.e22638.

229. Zvonova, E.A., Ershov, A.V., Ershova, O.A., Sudomoina, M.A., Degterev, M.B., Poroshin, G.N., Eremeev, A.V., Karpov, A.P., Vishnevsky, A.Y., Goldenkova-Pavlova, I.V. and Petrov, A.V., 2017. PASylation technology improves recombinant interferon-β1b solubility, stability, and biological activity. *Applied microbiology and biotechnology*, *101*(5), pp.1975-1987.