The role of histone methylation of H3K4 in polarization of macrophages stimulated by immune complex and lipopolysaccharide and its therapeutic potential in sepsis

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บทบาทของกระบวนการเติมหมู่เมทิลของฮิสโตน H3K4 ต่อการเปลี่ยนสภาพของแมโครฝาจที่กระตุ้นด้วยอิมมูนคอมเพลกซ์และไลโปพอลิแซ็กคาไรด์และศักยภาพในการบำบัดภาวะติดเชื้อในกระแสเลือด

น.ส.วิชญา เรือนใจมั่น

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ความยืดหยุ่นของแมโครฟาจเป็นกระบวนการหนึ่งที่แมโครฟาจสามารถเปลี่ยนสภาพระหว่างแมโครฟาจสองชนิด โดยขึ้นกับชนิดของสิ่งเร้าที่ได้รับ ซึ่งความยืดหยุ่นของแมโครฟาจเป็นกระบวนการสำคัญที่ส่งผลต่อการเกิดพยาธิสภาพในโรคต่าง ๆ หลายโรค เช่น โรคมะเร็ง โรคทางเมตาโบลิก โรคแพ้ภูมิตนเอง และโรคติดเชื้อ การควบคุมในระดับเหนือพันธุกรรมเป็นกลไกที่มีบทบาทสำคัญหนึ่งในการควบคุมกระบวนการนี้ แมโครฟาจที่ถูกกระตุ้นด้วยไลโปโพลีแซคคาร์ไรด์ (LPS) หรือ M(LPS) นำไปสู่การตอบสนองและหลั่งไซโตไคน์ที่เกี่ยวกับการอักเสบหลายชนิดในระดับสูง เช่น IL-12, TNFα และ IL-6 รวมทั้งมีการหลั่งไซโตไคน์ที่เกี่ยวข้องกับการต้านการอักเสบ IL-10 ในระดับที่ต่ำ ในขณะที่แมโครฟาจที่ถูกกระตุ้นด้วย LPS ร่วมกับสารประกอบแอนติบอดีแอนติเจน (IC) หรือ M(IC) มีการผลิตไซโตไคน์ที่เกี่ยวข้องกับการต้านการอักเสบ IL-10 ในระดับที่สูง และไซโตไคน์ที่เกี่ยวกับการอักเสบ IL-12 ในระดับต่ำเมื่อเทียบกับแมโครฟาจชนิด M(LPS) งานวิจัยนี้มีวัตถุประสงค์ในการศึกษาความยืดหยุ่นของแมโครฟาจที่เปลี่ยนสภาพจาก M(LPS) ไปเป็น M(IC) ในหลอดทดลอง รวมทั้งเปรียบเทียบรูปแบบของกระบวนการเติมหมู่ไตรเมทิลของฮิสโตน H3K4 (H3K4me3) ระหว่างแมโครฟาจทั้งสองชนิด โดยใช้แมโครฟาจเหนี่ยวนำมาจากเซลล์ไขสันหลังของหนูเมาส์ (BMDM) ผลการทดลองพบว่าแมโครฟาจมีความยืดหยุ่นที่สามารถทำให้เกิดการเปลี่ยนสภาพของแมโครฟาจจาก M(LPS) ไปเป็น M(IC) ในหลอดทดลองได้ หลังจากมีการพักเซลล์หลังจากการกระตุ้นด้วย LPS ก่อนการกระตุ้นครั้งที่สองด้วย LPS/IC เป็นเวลาสองวัน M(IC) ที่เปลี่ยนสภาพมาจาก M(LPS) มีระดับการเติมหมู่ฟอสเฟตของโปรตีนที่เกี่ยวข้องกับวิถีสัญญาณ MAPK, NF-KB และ PI3K/AKT ได้แก่ p38, p44/42, p65 และ Akt ในระดับใกล้เคียงกับ M(IC) ที่เปลี่ยนสภาพมาจากแมโครฟาจที่ไม่เคยถูกกระตุ้น (unstimulated) เพื่อศึกษารูปแบบของกระบวนการควบคุมในระดับเหนือพันธุกรรมระหว่าง M(LPS) และ M(IC) ผู้วิจัยได้ทำการศึกษาเพื่อเปรียบเทียบรูปแบบของ H3K4me3 ในแมโครฟาจทั้งสองชนิดโดยวิธี ChIP-seq ผลการทดลองพบว่า M(LPS) และ M(IC) มีความแตกต่างอย่างชัดเจนของระดับและรูปแบบ H3K4me3 ในระดับจีโนมโดยรวม โดยพบว่า M(IC) มีการเพิ่มขึ้นของ H3K4me3 ในขณะที่ M(LPS) มีการลดลงของ H3K4me3 นอกจากนี้ M(IC) ยังมีการเพิ่มขึ้นของ H3K4me3 ในบริเวณ *cis*-regulatory elements อีกด้วย และเมื่อศึกษาในระดับยีนจำเพาะพบว่า M(IC) มีการเพิ่มขึ้นของ H3K4me3 ในบริเวณโปรโมเตอร์ของยีนที่มีการรายงานว่ามีการแสดงออกเพิ่มขึ้นใน M(IC) ได้แก่ *Il10, Cxcl1, Csf3* และ *Il33* เมื่อเปรียบเทียบกับ M(LPS) และเพื่อศึกษาผลของการใช้ M(IC) ในการบำบัดรักษาโรค ผู้วิจัยได้ทำการศึกษาผลของการนำ M(IC) มาใช้ในการรักษาด้วยเซลล์ภูมิคุ้มกันบำบัด ในหนูทดลองที่มีภาวะติดเชื้อในกระแสเลือดจากการให้ LPS โดยพบว่ารูปแบบของการหลั่งไซโตไคน์ในหนูที่ได้ M(IC) มีการลดลงของระดับไซโตไคน์ที่เกี่ยวกับการอักเสบ IL-1β และ IL-p12p70 ในซีรัมเมื่อเปรียบเทียบกับหนูที่ได้รับแมโครฟาจที่ไม่เคยถูกกระตุ้น งานวิจัยนี้ได้แสดงให้เห็นถึงความสำคัญของการควบคุมโดย H3K4me3 ต่อการแสดงออกของยีนและการทำงานของแมโครฟาจชนิด M(IC) จะเป็นประโยชน์ในการออกแบบการควบคุมการทำงานของ M(IC) ในโรคต่าง ๆ และการประยุกต์ใช้ M(IC) ในการบำบัดและรักษาโรคในสัตว์ทดลองอีกด้วย

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Vichaya Ruenjaiman : The role of histone methylation of H3K4 in polarization of macrophages stimulated by immune complex and lipopolysaccharide and its therapeutic potential in sepsis. Advisor: Prof. TANAPAT PALAGA, Ph.D. Co-advisor: Asst. Prof. ASADA LEELAHAVANICHKUL, M.D., Ph.D.,Assoc. Prof. Yu-Wei Leu, Ph.D.

Macrophage plasticity is a process that allows macrophages to switch between two opposing phenotypes based on differential stimuli. Functional plasticity of macrophages contributes to disease pathogenesis such as cancer, metabolic diseases, autoimmune diseases and systemic infections. Epigenetics plays an important role in regulating this process. Macrophages stimulated with lipopolysaccharide (LPS) (M(LPS)) produce high level of pro-inflammatory cytokines such as IL-12, TNFα and IL-6 and low level of anti-inflammatory cytokine IL-10, while macrophages stimulated with LPS in the presence of immune complex (IC) (M(IC)) produce high level of IL-10 and low level of IL-12. In this study, we investigated the plasticity of M(LPS) to become M(IC) *in vitro* and compared the active histone mark (trimethylation on lysine 4 of histone 3 (H3K4me3)) between M(LPS) and M(IC) using murine bone marrow-derived macrophages. We found that macrophages exhibited functional plasticity from M(LPS) to M(IC) upon re-polarization after two days of resting *in vitro*. Phosphorylation of p38, p44/42, p65 and Akt in M(IC) re-polarized from M(LPS) was similar to M(IC) stimulated from resting macrophages. To obtain the epigenetic profiles of M(LPS) and M(IC), global H3K4me3 enrichments in both activated macrophages were compared. M(LPS) and M(IC) displayed marked differences in genome-wide enrichment of H3K4me3. M(IC) showed increased global enrichment of H3K4me3 whereas M(LPS) decreased enrichment when compared to unstimulated macrophages. Furthermore, M(IC) exhibited high H3K4me3 enrichment in all *cis*-regulatory elements. At individual gene, increased H3K4me3 enrichments were observed in the promoters of known genes associated with M(IC) including *Il10*, *Cxcl1*, *Csf3* and *Il33* when compared with M(LPS). Finally, to evaluate the therapeutic application of M(IC), we investigated the impact of M(IC) on systemic immune response by adoptive transfer of M(IC) in LPS-induced endotoxemia model. Cytokine profiles revealed that mice receiving an adoptive transfer of M(IC) acutely reduced the serum inflammatory cytokines IL-1β and IL-p12p70. This study highlighted the importance of epigenetics in regulating macrophages activation and functions of M(IC) that may influence macrophage plasticity and the potential therapeutic use of macrophage *in vivo*.

Field of Study:	Medical Microbiology	Student's Signature .....
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LPS	Lipopolysaccharide
IC	Immune complex
IL-12	Interleukin 12
IL-10	Interleukin 10
TNFα	Tumor necrosis factor alpha
IL-6	Interleukin 6
IFN-γ	Interferon γ
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
H3K4me3	Trimethylation on lysine 4 of histone 3
TLR	Toll-like receptor
BMDMs	Bone marrow-derived macrophages
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
L929	Mouse fibroblast cell
%	Percentage
w/v	Weight by volume

v/v	Volume by volume
PBS	Phosphate buffer saline
CD	Cluster of differentiation
DMSO	Dimethyl sulfoxide
ng	Nanogram
ml	Milliliter
h	Hour
min	Minute
IgG	Immunoglobulin G
OVA	Ovalbumin
ELISA	Enzyme-Linked Immunosorbent Assay
µl	Microliter
°C	Degree Celsius
HRP	Horse-radish peroxidase
rpm	Round per minute
mM	Millimolar
µg	Microgram
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate

mA	Milliampere
MAPK	Mitogen-activated protein kinase
NF-ĸB	Nuclear factor kappa B
µM	Micromolar
DNA	Deoxyribonucleic acid
ChIP	Chromatin Immunoprecipitation
bp	base pair
i.p.	Intraperitoneal injection
SEM	Standard error of the mean
FcγR	Fc gamma receptor
PAMP	Pathogen-associated molecular pattern
EAE	Experimental autoimmune encephalomyelitis
PRR	Pattern recognition receptor
MYD88	Myeloid differentiation primary response 88
TRIF	TIR-domain-containing adapter-inducing interferon-β
STAT	Signal Transducers and Activators of Transcription
IRF	Interferon-regulatory *factor*
MHC	Major histocompatibility complex
ITAM	Immunoreceptor tyrosine-based activation motifs

DC	Dendritic cell
APC	Antigen presenting cell
H3K27me3	Trimethylation on lysine 27 of histone 3
DAMP	Danger-associated molecular pattern
CEAS	*Cis*-regulatory Element Annotation System
PCA	Principal component analysis
TFBS	Transcription factor binding site
QIRI	Quantifies different datasets within specific region of interest
TSS	Transcription start sites
kb	Kilobase
IGV	Integrative Genomics Viewer
RNA	Ribonucleic acid

CHAPTER I

INTRODUCTIONS

Background

Macrophages are one type of white blood cells in the innate immune system that are found in most tissues. They respond to infection as the first line defense against pathogen (1). Macrophages can be divided into distinct specific subsets based on their functional phenotypes driven by distinct stimuli in their microenvironment. Macrophages play an important role in immune response and homeostasis with functional diversity such as inflammation, phagocytosis, regulation of metabolism, tissue remodeling and immunoregulation. However, a key feature of macrophages is the functional plasticity (2).

Macrophage plasticity is a process that allows them to switch between two different phenotypes depending on stimuli in microenvironment. Functional plasticity of macrophages contribute to various disease pathogenesis such as systemic infections, metabolic diseases, autoimmune diseases and cancer (3). Interferon γ (IFN-γ) and lipopolysaccharide (LPS)-stimulated macrophages or classically activated macrophage (M(LPS)) show pro-inflammatory activity which is characterized by production of high level pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-6, IL-1β and IL-12 (4). LPS and immune complex-stimulated macrophage (M(IC)) produces large amount of anti-inflammatory cytokine IL-10 while decreasing the pro-inflammatory cytokine IL-12, but produce high level of TNF, IL-6 and IL-1β (5, 6). IL-10 is a key multi-function regulatory cytokine regulating immune responses during infection and

dampening immune hyperactivation (7). IL-10 is produced by various immune cells including innate immune cells such as macrophages and adaptive immune cells such as T cell (8).

M(IC) or regulatory macrophage is categorized as non-classically activated macrophages that stimulated FcγR ligation by IgG complexes in occurrence of pathogen-associated molecular patterns (PAMPs) such as LPS or lipoteichoic acid through Toll-like receptors (TLRs). These activated macrophages show immunomodulatory activity due to the increased production of anti-inflammatory cytokine IL-10 and reduced pro-inflammatory cytokine IL-12 production (5). Moreover, RNA-seq data from the previous study reported that in transcriptional level M(IC) increased some genes expression and exhibit distinctive gene expression profile that is unique and does not overlap with M(LPS) or M(IL-4) (9). Therefore, M(IC) has potentials in dampening overt immune response and can be used in cellular therapy. In one study, adoptive transfer of M(IC) increased IL-10 production and reduced the disease severity in an animal model of multiple sclerosis (10). In another study, it was reported that endotoxemia mice receiving an adoptive transfer of M(IC) showed reducing disease severity. However, it remains unknown how stable the phenotype of M(IC) is upon transfer *in vivo*.

In macrophages, the expression of *Il10* is regulated by several transcription factors including Sp1, ERK and NF-κB (8, 11). In M(LPS), IL-10 is produced at low levels when its expression is regulated mainly by the NF-κB, MAPK and STAT pathways (12, 13). In contrast, FcγRs signaling activates Erk and p38 MAPK signaling in M(IC), resulting in the binding of transcription factor Sp1 to the *Il10* promoter (14).

Furthermore, IC/FcγR signaling also modulates the signaling downstream of TLR that regulates *Il12* transcription via PI3K/AKT signaling downstream leading to the decreasing of *Il12* expression in M(IC) (15).

Regulation of cytokines production in macrophages is regulated at several levels such as activation of transcription factors, epigenetic regulation and post-transcription regulation (2). In addition to the short live effect by transcription factors, the epigenetic regulation also plays a critical role in influencing long term plasticity by regulated cytokines production during the activation (16). Epigenetics regulate chromatin accessibility at the promoter and regulatory regions by several processes such as DNA methylation, histone modifications, and RNA-associated silencing (17). Various histone modifications such as methylation, acetylation and phosphorylation cause changes in chromatin structure to closed or opened chromatin, resulting in gene silencing or gene activating (18).

Histone methylations can be conducive or repressive for gene expression, depending on the type of the methylation and the locations of modified amino acids on the histone tails (19). Histone modifications at the promoter regions are associated with activating or silencing gene expression that regulates macrophage polarization (20, 21). Activation of Jmjd3, a histone H3K27 demethylase, resulted in the decreasing of repressive histone H3K27me3 and increasing chromatin accessibility leading to M(IL-4) signature gene expression and is crucial for regulating M(IL-4) development (21). H3K4me3 modification on certain genes encoding cell surface markers and chemokines is correlated with the transcriptional activity in monocyte-derived macrophages (20). These results together strongly indicated that histone H3 trimethylation on lysine 27 (H3K27me3) mark is correlated with inactive gene

promoters whereas histone H3 trimethylation on lysine 4 (H3K4me3) marks is correlated with actively transcribed genes and both play an essential role in polarization and activation in macrophages (20, 21).

Previous studies reported on the epigenetic regulation by histone modification for *Il10* expression in macrophages (22, 23). Macrophage-specific regulatory element DNase I hypersensitive sites (HSS–4.5) were identified in the mouse *Il10* locus. This site locates approximately 4.5 kb upstream of the transcription start site which is highly conserved and contains hyperacetylated histones and contains NF-κB binding site. The activation of HSS-4.5 is important for *Il10* expression in M(LPS) (11). The regulation of IL-10 production in M(IC) by histone modifications are also reported, the activation of ERK leads to increasing the phosphorylation of serine 10 on histone H3 at the *Il10* promoter, resulting in the recruitment of transcription factor SP-1 to *Il10* promoter and increases *Il10* expression (22). However, the global profile of H3K4me3 in M(IC), in comparison to M(LPS), has not been characterized. Gaining insight on the involvement of H3K4me3 in M(IC) may shed light on the plasticity and the stability of M(IC) phenotype *in vivo*.

Therefore, in this study, we first investigated the plasticity of M(LPS) and M(IC) *in vitro*. We next compared the profiles of H3K4me3 between M(LPS) and M(IC) to gain a comprehensive view on H3K4me3 involvement in the two distinctive macrophage phenotypes. Finally, the effect of M(IC) in systemic cytokine production was tested in LPS-induced endotoxemia model by adoptive transfer approach. The results obtained from this study may be beneficial in designing means to manipulate macrophages function for therapeutic purpose in the future.

Research question

Does histone methylation of H3K4me3 regulate IL-10 production in M(IC) and affect its plasticity?

Hypothesis

IL-10 production in M(IC) is regulated by histone methylation of H3K4me3 and M(IC) can be therapeutically applied.

Objectives

1. To examine the plasticity between M(LPS) and M(IC) *in vitro.*
2. To investigate the histone methylation profiles of H3K4me3 in M(LPS) and M(IC).
3. To test the therapeutic potential of M(IC) in sepsis mouse model.

CHAPTER II

LITERATURE REVIEW

Macrophages and macrophage subsets

Macrophages are one type of white blood cells in the innate immune system, originating from myeloid precursor cells (24). Macrophages are referred to by various names such as microglia, Kupffer cells and osteoclasts depend on its location. They are phagocytes that can be found in most tissues and respond to infection as the first line defense against pathogen (1). Mouse macrophages are characterized and identified by the expression of several surface markers such as F4/80 and CD11b.

Macrophages can be divided into distinct specific subsets based on their functional phenotypes that are driven by distinct stimuli in their microenvironment. The functions of macrophages in pro-inflammatory responses, scavenging, remodeling potential and anti-inflammatory activity are mediated by distinct subsets of macrophages (25). Interferon γ (IFNγ) and lipopolysaccharide (LPS)-stimulated macrophages or classically activated macrophages (M(LPS) or M1) exhibit pro-inflammatory activity, phagocytosis microbes, and initiate immune response which is characterized by production of high levels pro-inflammatory cytokines such as interleukin-12 (IL-12), tumor necrosis factor (TNF), IL-6 and IL-1β and the production of low levels anti-inflammatory cytokine IL-10 (4). IL-4-stimulated macrophages or alternatively macrophages (M(IL-4) or M2) play a role in regulating wound healing and tissue repair. In contrast with M(LPS), LPS and immune complex-stimulated macrophage (M(IC)) produces large amount of anti-inflammatory cytokine IL-10 while

decreases the pro-inflammatory cytokine IL-12. However, M(IC) maintains the production of high levels of TNF-α, IL-6 and IL-1β (5, 6).

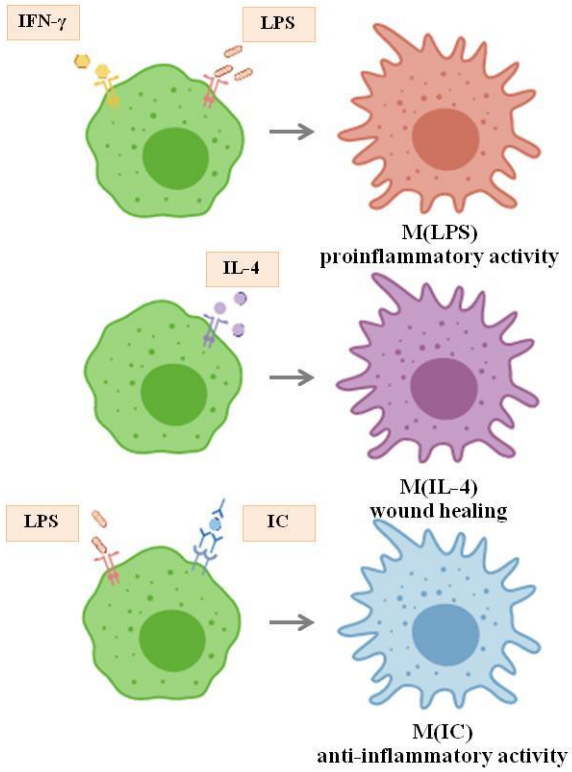


Figure 1| Macrophages phenotypes and their stimuli

A Schematic diagram shows different subsets of macrophages and the stimuli. IFN-γ and LPS stimulate macrophage to M(LPS) that have pro-inflammatory activity. IL-4 activates macrophage to M(IL-4) that regulate wound healing. LPS in the presence of immune complex stimulate macrophages to become M(IC) that exhibit anti-inflammatory phenotype.

Macrophage plasticity

Macrophages play an important role in immune response and homeostasis with functional diversity such as inflammation, phagocytosis, proliferation, regulation of metabolism, tissue remodeling and immunoregulation. One of the key features of macrophages is functional plasticity (2). Macrophage plasticity is a process that allows them to switch between two different phenotypes depending on the stimuli and their microenvironment. Functional plasticity of macrophages contributes to various disease pathogenesis, including cancer, metabolic diseases, autoimmune diseases and systemic infections (3). For instance, a switch of macrophage polarization from M1 to M2 occurs in chronic phase of infection leading to inability to clear pathogen (3) while a switch of macrophage polarization from M2 to M1 can found in Atherosclerosis resulting in an inflammatory response in the blood vessel (26). The pathology is frequently associated with macrophage plasticity that allows dynamic change between M(LPS) and M(IL-4) (3). In obesity, IL-4 released from adipocytes is believed to polarize macrophages to M(IL-4) and prevent overt inflammation in lean tissue. While in obese tissues, an increase in infiltration of macrophages is observed. Profiling these adipose tissue macrophages (ATMs) indicated that they exhibit M(LPS) characteristics such as upregulation of inflammatory markers TNF-α and inducible nitric oxide synthase (iNOS) (2). However, it remains unclear whether macrophages have functional plasticity between M(LPS) and M(IC).

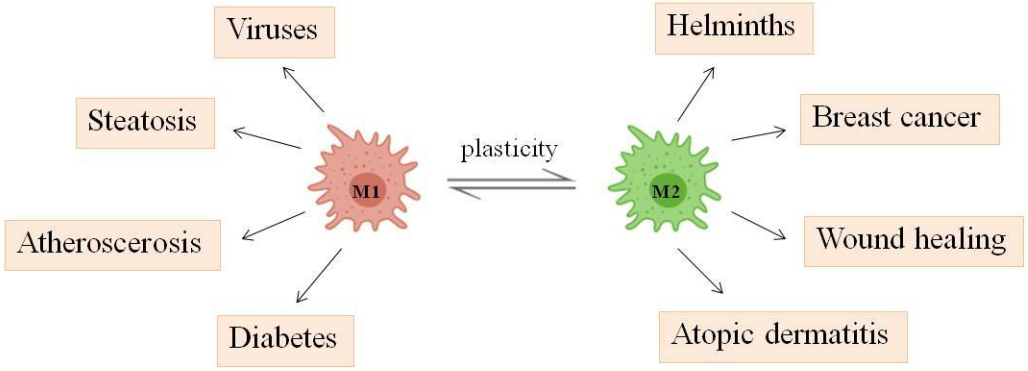


Figure 2 | The plasticity between M(LPS) (M1) and M(IL-4) (M2) in diseases progression

Dynamic changes of macrophage phenotypes switching between M1 and M2 occur in several diseases including infectious diseases, metabolic disease, autoimmune disease and cancer.

Regulatory macrophage (M(IC)) and its therapeutic potential

M(IC) or regulatory macrophage is categorized as non-classically activated macrophages that are stimulated by FcγR ligation with IgG complexes in the presence of pathogen-associated molecular patterns (PAMPs) such as LPS or lipoteichoic acid through Toll-like receptors (TLRs) (5). These activated macrophages show immunomodulatory activity due to the increased production of anti-inflammatory cytokine IL-10 and reduced pro-inflammatory cytokine IL-12 production (5). M(IC) exhibits distinctive gene expression profile that is unique and does not overlap with M(LPS) or M(IL-4) (9). Moreover, RNA-seq data from the previous study reported that at the transcriptional level, M(IC) increased unique gene expression, including *Il10, Lif, Il33, Ildr1, Flrt3, Xcr1* and *Odc1* when compared to M(LPS) (9). Therefore, M(IC) has potential in preventing immune hyperactivation and can be used in cellular therapy. In one study, adoptive transfer of M(IC) reduced the severity of experimental autoimmune encephalomyelitis (EAE) an animal model of multiple sclerosis (10). In another study, it was reported that mice receiving an adoptive transfer of M(IC) showed reducing disease severity of sepsis (9). To divert M(LPS) to produce higher amount of IL-10, the signaling downstream of IC/FcγR can be replaced with other stimuli such as PGE2, GPCR ligand, glucocorticoid, apoptotic cell and IL-10 (9, 24).

TLR4 signaling pathway in macrophages

TLR4 is a prototype of pattern recognition receptors (PRRs) that recognizes LPS from Gram negative bacteria. TLR4 ligation results in the recruitment of the adaptor molecules Myeloid differentiation primary response 88 (MYD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) (27). They are critical for the activation of subsequent downstream signaling pathways and gene expression. Among these, Nuclear factor kappa B (NF-κB) , Signal Transducers and Activators of Transcription (STAT), interferon-regulatory *factor* (IRFs) and Activator protein 1 (AP1) (1) are activated, leading to robust production of pro-inflammatory cytokines (e.g. IFN-γ, IL-12, TNF, IL-6, and IL-1β), chemokines (e.g. C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 11 (CXCL11), and CXCL10), and antigen presentation molecules (e.g. MHC members), co-stimulatory molecules and antigen-processing peptidases (1). Additionally, IFN-γ priming leads to remodeling of chromatin structure to a more accessible status for TLR-induced transcription factors binding resulting in the increasing TLR-induced gene expression (28).

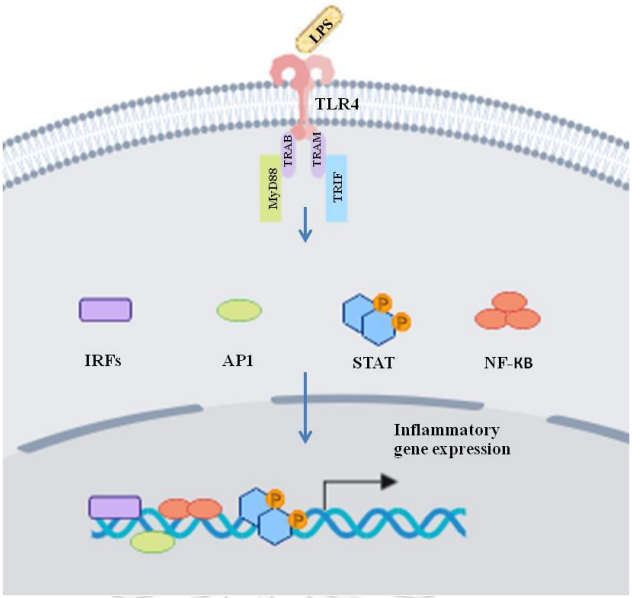


Figure 3 | TLR4 and its downstream signaling

The binding of TLR4 and LPS recruits MyD88 and TRIF leading to the activation of MAPK, NF-ĸB, STAT and IRFs, which regulated in the expression of inflammatory genes.

FcγR signaling pathway in macrophages

Immune complex (IgG) can activate macrophages through FcγR. FcγR ligation triggers the activation of src family kinase-mediated phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs). This activation recruits of the tyrosine kinase Syk, signal transduction molecule Ras and phosphoinositide 3-kinase (PI3K) for activation of downstream pathways including FCγR-associated genes expression, the activation of phospholipase C, generation of intracellular calcium flux, and NADPH activation (29).

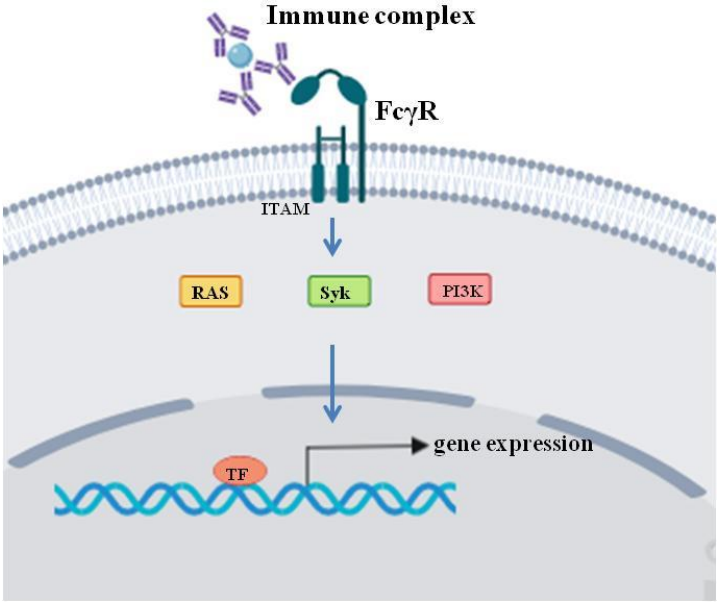


Figure 4 | FCγR and its downstream signaling

FcγR ligation by immune complex activates downstream signaling pathway RAS, Syk and PI3K. The activation triggers the recruitment of transcription factors to bind to gene promoter, resulting in FcγR-associated genes expression.

Cross talk between TLR4 and FCγR signaling in M(IC)

In M(IC), both the activation of TLR4 and FCγR ligation trigger the activation of macrophages to anti-inflammatory phenotype in contrast with TLR4 activation alone. Signaling from immune complex alone stimulates macrophages to produce baseline activation of inflammatory mediators (30) while the stimulation of macrophages with LPS together with immune complex increases the production of regulatory cytokine IL-10 and reduces IL-12 production. The signaling from TLR4 and FCγR trigger the activation of extracellular signal-regulated kinases 1 and 2 (Erk) and p38, the components of MAPK signaling pathway, resulting in increased of *Il10* and decreased *Il12* (31). Since IL-10 is an immunomodulatory cytokine that regulates immune response which is important to control inflammatory process, the regulation of IL-10 expression in M(IC) provides a target for therapeutic intervention.

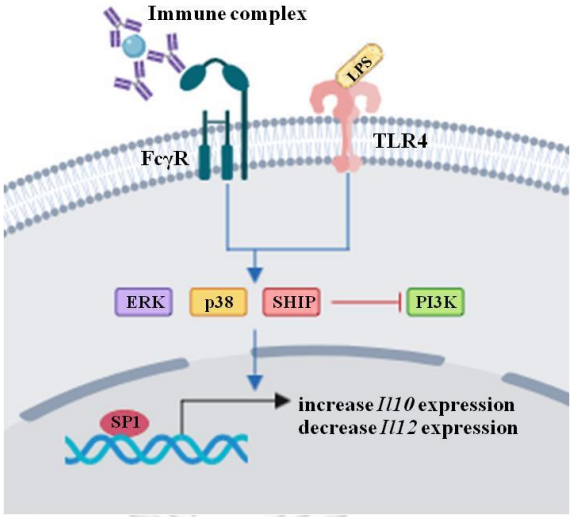


Figure 5 | Cross talk between TLR4 and FCγR signaling in M(IC)

FcγR ligation with TLR4 signal by immune complex and LPS activates downstream signaling pathway such as MAPK signaling pathway. The activation triggers the increasing of *Il10* and decreasing of *Il12* expression in M(IC).

IL-10 and its functions

IL-10 is a key multi-function regulatory cytokine that modulates immune response during infection with viruses, bacteria, fungi, protozoa and helminths and prevents hyperactivation of an immune response (7). IL-10 is produced by various cell types of both innate immune cells including macrophages, monocytes, dendritic cells (DCs), mast cells, neutrophils, eosinophils and natural killer cells and adaptive immune cells including T cells and B cells (8, 32). It functions in promoting B cells functions by enhancing proliferation, survival, differentiation and antibody production and promoting regulatory T cells functions by enhancing IL-10 production. For inhibitory functions, IL-10 inhibits functions of antigen presenting cells (APCs) and downregulates the expression of MHC class II and costimulatory molecules (33). IL-10 is the major cytokine that is produced by M(IC) and considered to be a functional cytokine signature of M(IC) (34)

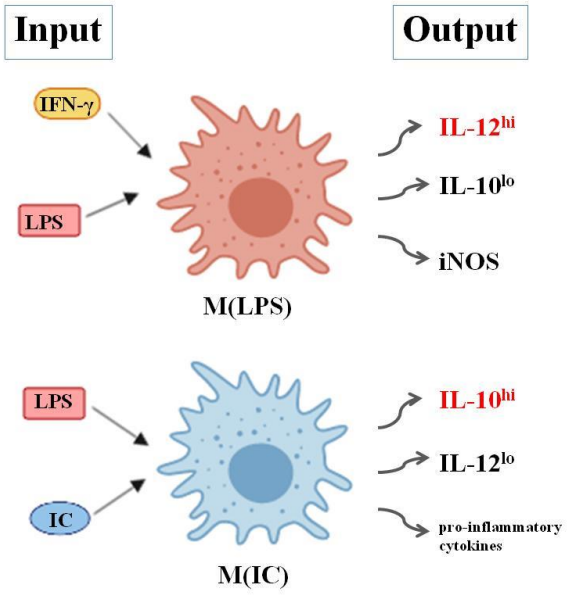


Figure 6 | Difference in stimuli and cytokines production in M(LPS) and M(IC)

A Schematic diagram showed different stimuli and cytokines production in M(LPS) and M(IC). IFN-γ/LPS-stimulated macrophages (M(LPS)) produce high levels of IL-12 and low levels of IL-10 while M(IC) that stimulated by LPS/IC produce high levels of IL-10 and low levels of IL-12.

Regulatory signaling of IL-10 expression in macrophages

In macrophages, the expression of *Il10* is regulated by several transcription factors including Sp1, ERK, p38 and NF-κB (8, 11). In M(LPS), IL-10 is produced at low levels where its expression is regulated mainly by several pathways including NF-κB, MAPK and STAT pathways, resulting in low levels of IL-10 production (12, 13). In contrast, FcγRs signaling activates ERK and p38 MAPK signaling in M(IC), resulting in more chromatin accessibility for the transcription factor Sp1 in the *Il10* promoter (14). Furthermore, IC/FcγR signaling also modulates the signaling downstream of TLR that regulates *Il12* transcription via PI3K/AKT signaling downstream leading to the decreasing *Il12* expression in M(IC) (15).

The epigenetic regulation of gene expression by histone modification

The regulation of macrophage polarization and plasticity are regulated at several levels such as activation of transcription factors, epigenetic regulations and post-transcription regulation (2). In addition to the short live regulatory effect by the transcription factors, the epigenetic regulations also play a critical role in influencing long term response by regulating cytokines production during the activation (16). Epigenetics regulated gene expressions that are not encoded in the DNA sequence. Epigenetics cause the modification of chromatin accessibility at the promoter and the regulatory regions by several processes such as DNA methylation, histone modifications, and RNA-associated silencing (17).

Histone modification is a dynamic modification of histone proteins by histone modifying complexes that catalyze the addition or removal of various chemical

elements on the histones tail. Various histone modifications such as methylation, acetylation and phosphorylation cause changes in the chromatin structure or chromatin remodeling resulting in closed or opened chromatin. These regulations culminate in gene silencing or gene transcription depending on the type of histone mark (18).

Histone modifications play a role in several biological processes including transcriptional activation/repression, chromosome packaging and repair of DNA damage. Histone H3 is the most modified histones by acetylation at lysine 9, 14, 18, 23, and 56, methylation at lysine 4, 9, 27, 36, and 79 and arginine 2, and phosphorylation at ser10, ser28, Thr3, and Thr11. Histone H4 can be modified by acetylated at lysine 5, 8, 12 and 16, methylation at arginine 3 and lysine 20, and phosphorylation at serine 1 (35). Histone acetylation and phosphorylation are generally associated with transcriptional activation while histone methylations are associated with both gene activation and gene silencing depending on the locations of modification and the type of the methylation on the histone tails (35, 36).

Regulating of macrophage polarization by histone modifications

Histone modifications at the promoter regions are associated with activating or silencing gene expression that regulates macrophage polarization (20, 21). Previous study reported on the regulation of macrophage polarization by repressive histone mark H3K27me3 (21). Activation of Jmjd3, a histone H3K27 demethylase function in removing the methyl group from lysine 27 on the histone H3, resulted in decreasing of H3K27me3 and increasing chromatin accessibility in M(IL-4). *Jmjd3* KO macrophages exhibited defects in M(IL-4) polarization (21). H3K4me3 modification on the regulatory regions of genes encoding cell surface markers and chemokines is correlated

with the transcriptional activity in monocyte-derived macrophages (20). These results together strongly indicated that trimethylation of histone H3 on lysine 27 (H3K27me3) is correlated with inactive gene promoters whereas trimethylation of histone H3 on lysine 4 (H3K4me3) is correlated with actively transcribed genes and both play an essential role in polarization and activation in macrophages (20, 21).

Previous studies reported on the epigenetic regulation by histone modification for *Il10* expression in macrophages (22, 23). Macrophage-specific regulatory element DNase I hypersensitive sites (HSS–4.5) were identified in the mouse *Il10* locus. This site locates approximately 4.5 kb upstream of the transcription start site which is highly conserved and contains hyperacetylated histones and contains NF-κB binding site. The activation of HSS-4.5 is important for *Il10* expression in M(LPS) (11). The regulation of IL-10 production in M(IC) by histone modification are also reported where ERK activation leads to phosphorylation of serine 10 on histone H3 (H3ser10) at the *Il10* promoter. This event increases the recruitment of transcription factor SP-1 to *Il10* promoter and increases *Il10* transcription (22).

Histone Methylation and histone demethylation

Methylation of lysine and arginine residues in the histone tails is crucial to chromatin modification that influences biological process during development and cellular responses (19). Histone can be mono (me1), di(me2), or tri(me3) methylated on lysine and can be mono(me1), symmetrically dimethylated (me2s), or asymmetrically dimethylated (me2a) on arginine of the histone tail (37). Methyl groups are believed to have slow turnover rate more than other post-translational modification

(PTMs) (38). Methyltransferases and demethylases function in addition and removal of methyl groups from different amino acid residues on histones tail respectively (19). Some types of histone methylation are needed to be stably maintained such as methylation involved in the inheritance through mitosis of a silenced heterochromatin state whereas others may have to be able to change such as methylation involved in cells differentiate or respond to an environmental cue. Different methylation at lysine residues on the histone tails displays differential turnover rates (39).

The active histone H3K4me3

H3K4me3 is the tri-methylation of the fourth lysine from the N-terminus of histone H3. This histone modification is one of the most studied histone modifications that are associated with the promoters regions (40). H3K4me3 marks positively correlated with gene expression in active genes (40). This modification recruits the chromatin remodeling factors Chromodomain-helicase-DNA-binding protein 1(CHD1) and Nucleosome-remodeling factor subunit BPTF(BPTF) while preventing the binding of the repressive Nucleosome Remodeling Deacetylase (NuRD) and the inhibitor of acetyltransferase (INHAT) complexes, resulting in chromatin remodeling to opened chromatin (41-43). In macrophages, H3K4me3 modification on the regulation regions is associated with the transcriptional activity of many genes (20). However, the profile of H3K4me3 in M(IC) is still undocumented.

ChIP-sequencing

Chromatin immunoprecipitation (ChIP) followed by next-generation sequencing (ChIP-seq) is a technique for genome-wide profiling of histone modifications and DNA-binding proteins (44). In a ChIP experiment, DNA binding protein which is interested histone modifications is investigated using antibody to the modified histone and immunoprecipitate the DNA-protein complex. After precipitation, crosslink of DNA and proteins are reversed and DNA is purified for sequencing. ChIP-seq provides higher resolution and greater coverage data than ChIP-chip that improves the characterization of transcription factor binding sites and empowers sequence motifs identification (44). ChIP-seq produces large amount of data and effective computational analysis that crucial for unmasking epigenetics mechanisms.

Sepsis and immune response to sepsis

Severe sepsis is a common life-threatening condition that is one of the most common causes of deaths from infection. Patient with sepsis has extreme immune reaction toward infection in the bloodstream causing leaking of blood vessel, systemic inflammation, leading to multi-organ dysfunction and death(45).

Patients usually present with fever, shock, and respiratory failure from an uncontrolled pro-inflammatory response. The immune responses initially mediated by the activation of PRRs such as TLRs by PAMPs and danger-associated molecular patterns (DAMPs) originating from the infection (46). In the primary phase after the infection, the activation of PRRs results in high production of pro-inflammatory

cytokines and chemokines including TNF-α, IL-1β, IL-12 and IL-6 (47). This hyper-inflammatory phase has been termed a “cytokine storm that drive systemic inflammation. This phase is the major cause of death in sepsis and it is a target for therapeutic intervention. Following this phase, some patients may develop an immunosuppressive response, resulting in increased host susceptibility to secondary bacterial infections.

Nowadays, sepsis is a severe multi-system complication with difficult treatments. The effectiveness of the treatment in sepsis depends on the time of diagnosis, immediately starting the antibiotic treatment and patient’s underlying disease (48). Antibiotics are mostly common treatment using during sepsis. However, the increasing antibiotic resistance and delay diagnosis leads to insufficient or incorrect treatment (48). Presently, there are many clinical studies to develop a new treatment strategy that more specific determinants for sepsis treatment. Most of these approaches aim to modulate the immune response to preventing sepsis physiopathology and multiple organ failure (48). For example, some studies used vasoactive agents, Corticosteroids and anti-endotoxin treatment for sepsis therapies (49, 50). However, there are insufficient data on clinical usage and need further studies to develop a new treatment strategy.

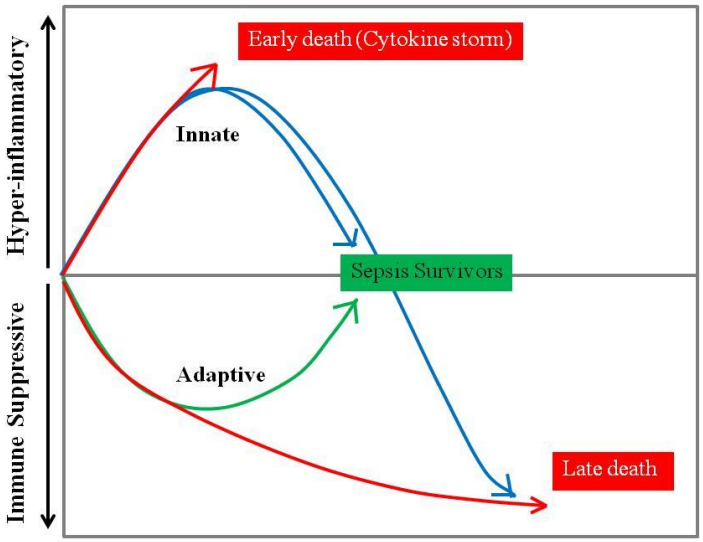


Figure 7 | Immune response to sepsis

The immune response in sepsis initials with hyper-inflammatory phase by high production of pro-inflammatory cytokines and chemokines. After this phase, patients will develop to immunosuppressive phase leading to secondary infections.

As mentioned before, previous study reported on using M(IC) adoptive transfer in mice for therapeutic approach (10). The adoptive transfer M(IC) protected mice from lethal endotoxemia by showing 90% survival when compared to the adoptive transfer with resting macrophage (9). Therefore, the therapeutic application of adoptive transfer M(IC) may be a new way for treatment diseases-associated with inflammation such as sepsis.

CHAPTER III

MATERIALS AND METHODS

Mice

Six to eight-week-old female C57BL/6 mice were purchased from Nomura Siam International (Bangkok, Thailand). All procedures were reviewed and approved by Chulalongkorn University Animal Care and Use Committee (CU-ACUP 024/2558). All procedures involved lab animals were performed in accordance with regulations issued by Chulalongkorn University IACUC.

Murine macrophages

Bone marrow-derived macrophages (BMDMs) were isolated from tibias and femurs by flushing with Dulbecco's Modified Eagle Medium (DMEM) (HyClone, USA) supplementing with 10% (v/v) fetal bovine serum (FBS) (GIBCO, USA), 1% (w/v) sodium pyruvate (Hyclone), 1% (w/v) HEPES (Hyclone), 100 U/ml penicillin, 0.25 mg/ml streptomycin (Hyclone), 20% (v/v) L929 cell–conditioned media and 5% (v/v) horse serum (Hyclone) in Forma Direct Heat CO_2 Incubator HEPA class 100 (Thermo Electron corporation, USA) at 37°C, 5% CO_2 on non-tissue culture treated plates. Cells were cultured for 4 days before added 3 ml of fresh DMEM media supplemented with 20% (v/v) L929-conditioned media and 5% (v/v) horse serum. Cells were harvested at day 7 using cold PBS. BMDMs were plated 24 h before use. Macrophages were confirmed by the expression of F4/80 and CD11b by flow cytometer.

BMDMs frozen stock preservation and thawing

At day 7, BMDMs were harvested by cold PBS and centrifuged at 1000 rpm, 5 min. Cells were gently resuspended in one part of 80% DMEM media supplemented with 20% FBS and one part of 60% DMEM media supplemented with 20% FBS and 20% DMSO. Frozen stock of cells were kept at -80°C until use. BMDMs were rapidly thawed at 37°C in a water bath and centrifuged in cold serum free media at 1000 rpm, 5 min. Cells were resuspended in 8 ml of DMEM media supplemented with 20% L929-conditioned media and 5% horse serum. Cells were plated on non-tissue culture treated plates for 3 days before use.

Preparation of M(LPS) and M(IC)

BMDMs were primed with 10 ng/ml of recombinant mouse IFN-γ (BioLegend, USA) for 18 h before stimulation. M(LPS) and M(IC) were generated by stimulating with 100 ng/ml of Salmonella LPS (Sigma-Aldrich, USA) or 100 ng/ml of LPS in combination with immune complex. Immune complex was prepared in 10-fold molar excess of rabbit anti-OVA IgG (Sigma-Aldrich) against OVA (Sigma-Aldrich) and incubated for 30 min at room temperature (51). Ratio volume of immune complex to media is 1:100 (v/v) were used for stimulation. For an *in vivo* adoptive transfer study, $5x10^6$ cells of BMDMs were plated on 10 cm treated plate before stimulated as described above for 4 h. For ChIP assay, $10x10^6$ cells of BMDMs ($5x10^6$ cells/plate) were plated on 10 cm treated plate before stimulated as described above for 4 h

Repolarization of macrophages

BMDMs ($2.5x10^5$ cells/well) were stimulated without IFN-γ priming to M(LPS) using 100 ng/ml of Salmonella LPS. After 24 h, cells were washed with warm media and rested in culture media for 48 h before re-polarization to M(IC) by stimulated with 100 ng/ml of LPS in combination with immune complex. Cultured media were harvested at 24 h after stimulation to measure IL-10 and IL-12p70 by Enzyme-Linked Immunosorbent Assay. M(LPS) and M(IC) were used as a negative control. For Western blot analysis, BMDMs were stimulated without IFN- γ priming to M(LPS) for 24 h, cells were washed with warm media and rested in culture media for 48 h or 2 h before re-polarization to M(IC) as described above. Protein lysates were harvested at 0, 5, 15 and 30 min after stimulation and M(IC) stimulated from naïve BMDMs were used as a control.

Enzyme-Linked Immunosorbent assay (ELISA)

Culture supernatants from BMDMs treated as indicated were harvested and subjected to measurement of IL-10 using ELISA max™ standard set (Biolegend) and IL-12p70 using purified rat anti-mouse IL-12 p40/p70 (coating antibody) and biotin rat anti-mouse IL-12 p40/p70 (detecting antibody) (BD Biosciences, USA) (Culture supernatants were collected after treatment as indicated and kept at -80°C until use. ELISA was performed following the manufacturer's protocol. Briefly, one hundred µl/well of anti-IL-12p40 antibody or anti-IL-10 antibody in coating buffer was coated as capture antibodies for overnight at 4°C. Unbound antibodies were removed and the wells were washed with 200 µl/well of washing buffer for 5 times. Two hundred µl of

Blocking buffer (10% (v/v) FBS in PBS) was added and plates were incubated for 1 h at room temperature. After Blocking buffer was removed and washed as described above, 100 µl of diluted samples (1:20 to 1:50, IL-12p70 and undiluted to 1:2, IL-10) and standards (Biolegend) (prepared in 2-fold serial dilutions) were added and incubated for 1 h (IL-12p70) or 2 h (IL-10) at 4°C. Following this step, wells were emptied and washed as described above, 100 µl of biotinylated anti-IL-12p40 antibody or anti-IL-10 antibody was used for detection antibodies and incubated for 1 h at room temperature. After that, wells were emptied and washed as described above, 100 µl of streptavidin-conjugated HRP was added and incubated for 30 min at room temperature. Wells were washed with 200 µl/well of washing buffer for 6 times. TMB substrate solution (100 µl/well) was added and incubated in the dark for 2-20 min at room temperature. Finally, the reactions were stopped by addition of 100 µl of 2N H_2SO_4. The signals were detected by Multiskan FC (Thermo scientific, USA) at OD_{450}.

Western blot

BMDMs were treated as indicated and the cell lysates were collected. Cells were washed twice using cold PBS and proteins were extracted by adding 40 µl of ice-cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10% Sodium Deoxycholate, 20% SDS containing Phosphatase inhibitor from Cell Signaling Technology and mini pack Protease Inhibitor from Roche Diagnostics, USA). Cell lysates were transferred to new 1.5 ml microcentrifuge tubes (Axygen Scientific, USA) and mixed by vortex mixer model G560E (Scientific Industries, USA) for 10 seconds. Samples were cleared by centrifugation at 10,000 rpm 4°C for 10 min using Centrifuge

5424 R (Eppendorf, Germany). The supernatants were collected and measured for protein concentration by using BCA Assay Protein Assay kit (Pierce, USA). Protein lysates were kept at -80°C until use.

Twenty μg of protein was added with forty μl of 2x loading dye (100 mM Tris pH 6.8, 4% (w/v) SDS, 20% (v/v) Glycerol, Bromphenol blue containing 10% (v/v) β-mercaptoethanol from Sigma-Aldrich) and boiled at 100°C for 5 min on Thermomixer Compact (Eppendorf). An equal amount of loading volume was loaded on 8% SDS-PAGE. Proteins were separated at 100 volts for 90 min by using Protein III system (Biorad, USA) and running buffer.

Proteins were transferred to PVDF membranes (Millipore, USA) by using semi-dry transfer Trans-Blot SD (Biorad) in transfer buffer (Appendix A) for 90 min with a current at 80 mA for 1 gel and 160 mA for 2 gels. The PVDF membrane was blocked with 3% skim milk (BD Biosciences) in PBS-Tween20 (0.1%) for 5 min twice and probed with primary antibodies at 4°C overnight. The antibodies used were as follows: rabbit anti-phospho-NF-κB p65 (1:2000), rabbit anti-NF-κB p65 (1:4000), rabbit anti-phospho-Akt (1:2000), rabbit anti-Akt (1:4000), rabbit anti-phospho-p38 (1:2000), rabbit anti-p38 (1:4000), rabbit anti-phospho-ERK1/2 (p44/42) (1:2000), rabbit anti-ERK1/2 (p44/42) (1:4000), rabbit anti-phospho-SAPK/JNK (1:2000), rabbit anti-SAPK/JNK (1:4000) (Cell Signaling Technology, USA), and mouse anti-β-actin (1:10000) (Chemicon-Millipore, USA). The membranes were incubated for 1 h at room temperature with shaking and washed for 5 min twice and 10 min three times. After washing, secondary antibody HRP-conjugated donkey anti-rabbit IgG (1:4000) or HRP-conjugated sheep anti-mouse IgG (1:4000) (Cell Signaling Technology) were

added and the membranes were incubated for 1 h at room temperature with shaking before washing as described above.

For developing the chemiluminescent signal, the solution A (100 mM Tris-HCl, pH 8.5, 196.5 μM coumaric acid and 1.24 mM luminal) was mixed with solution B (100mM Tris-HCl pH 8.5 and 0.009% H2O2) and added on PVDF membrane and incubated for 1 min. The membranes were wrapped in the plastic wrap and placed in Hypercassette (Amersham Biosciences, UK) to expose to High Performance Chemiluminescence Film Amersham HyperfilmTM ECL (Amersham Biosciences) in the dark. The X-ray film was developed in developer solution, washed with water, fixed in fixer solution and washed again with water before allowing to air dry.

ChIP-seq and data analysis

BMDMs were stimulated using the procedure described above. The enrichment of H3K4me3 on specific DNA regions were investigated using SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology) following the manufacturer's protocol as showed in Fig 8. Briefly, 37% formaldehyde was used to preserve the cross-link between proteins and DNA located in close proximately. Cells were lysed by treating with nuclear lysis buffer. Lysed cells were sonicated to shear nuclei and treated with micrococcal nuclease to shear chromatins. Genomic DNA lengths were optimized to be in the range of 150-1000 bp. Rabbit anti-H3K4me3 antibody (Cat#9751S Cell Signaling Technology) were added and incubated at 4°C overnight. Protein G-conjugated magnetic beads were added and incubated at 4°C for 2 h. Magnetic beads were separated and washed with washing buffer. ChIP Elution buffer were added, and the reaction was incubated at 65° for 2 h

to reverse cross linking of protein/DNA complex. The supernatants were collected and purified for DNA sequencing.

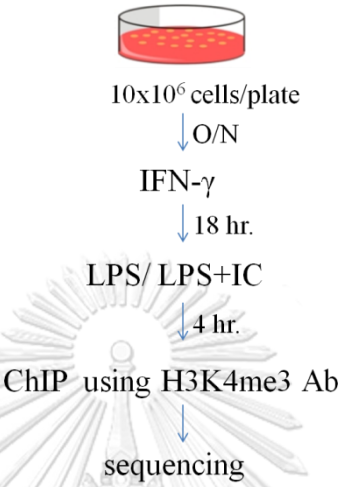


Figure 8 | The experiment set up for ChIP

The protocol used to investigate the histone methylation of H3K4me3 in M(IC) by ChIP-seq

ChIP-seq were performed using 50 bp single-end reads sequencing by BGI (Beijing, China). Overall ChIP-seq data analysis protocol is depicted in Fig 9. Trimmed sequences were examined for its quality by FastQC (52) and aligned to the reference genome by Bowtie2 (53) (more than 97% mapped). Regions of enrichment were found using MACS14 and MACS2 (54). Circos (55) and Venn diagram (56) were employed to visualize the designated ChIP-enrichment within globally. Epigenomic correlation was evaluated by TCOR in EpiMINE (57). CEAS (58) were used to reveal enrichment in *cis*-regulatory regions. IGV (59) and QIRI in EpiMINE were used to visualize and quantify the enrichment in target genes. MEME-ChIP (60) was used to identify the

novel enrichment motifs and possible associated regulators/transcription factors. In the end, the possible regulatory signaling pathways and networks were revealed by KEGG pathway analysis (61).

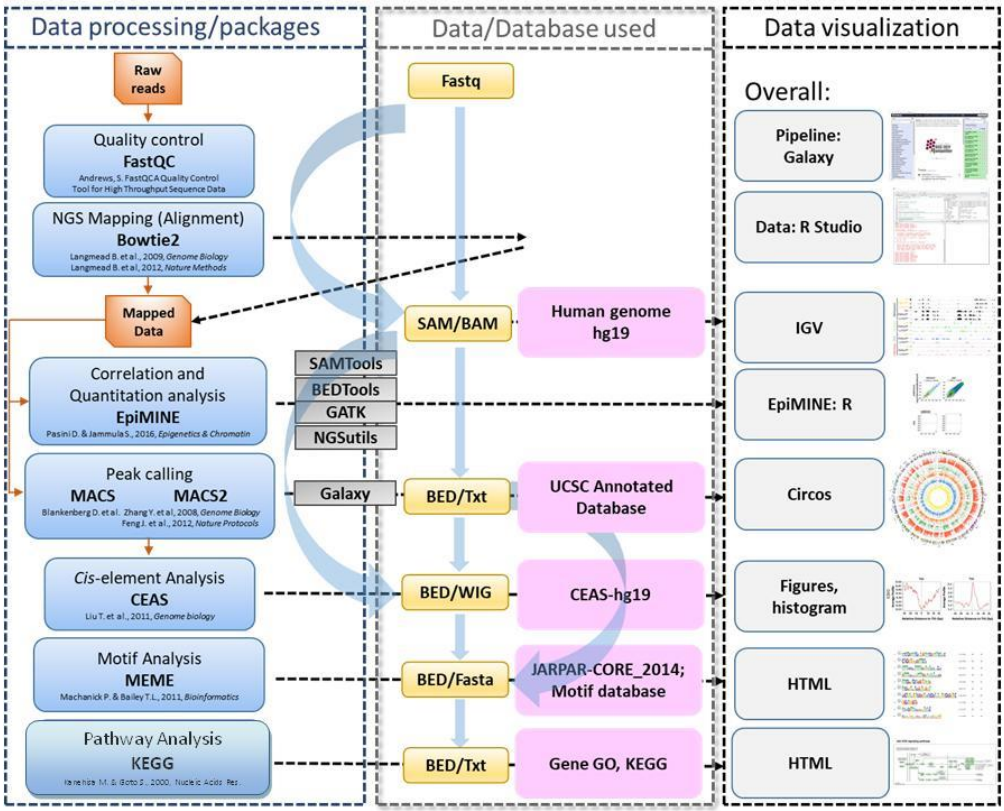


Figure 9 | ChIP-seq data analysis pipeline

Pipeline displayed the lists of used Bioinformatics packages (left panel), database (center) and visualization tools (right).

Repolarization of macrophages

BMDMs ($2.5x10^5$ cells/well) were treated with inhibitors are IOX-1 (pan H3 histone demethylase inhibitor) and JIB-04 (H3K4me3 demethylase inhibitor) at 66 mM and 1.71 mM (2xIC50) for 30 min before polarization to M(LPS) using 100 ng/ml of Salmonella LPS. After 24 h, cells were washed with warm media and rested in culture media for 2h before re-polarization to M(IC) using 100 ng/ml of LPS in combination with immune complex. Cultured media were harvested at 24 h after stimulation to measure IL-10 and IL-12p70 by Enzyme-Linked Immunosorbent Assay. M(LPS) and M(IC) without re-stimulation were used as a negative control.

Adoptive transfer of M(IC)

8 week-old of female C57BL/6 mouse (n=4 per group) was adoptive transferred with $1x10^6$ cells of M(IC) or unstimulated macrophages by *i.p.* route 3 h before E. coli LPS (Sigma-Aldrich) challenged (sublethal dose, 1 mg/kg body weight, *i.p.* route) to induce endotoxemia. Blood was collected at 1 and 6 h after the LPS challenge and used to detected cytokines by Bio-plex assay.

Bio-Plex assays

Blood serum collected from endotoxemia mice as described above was used to measure cytokines IL-1β, IL-12p70, IL-6, TNFα, IL-17, IL-10 and IL-4 by using Bio-Plex Pro™ Mouse Cytokine 7-plex assay kit (Bio-Rad) following the manufacturer's protocol. This experiment used anti- IL-1β, anti- IL-4, anti- IL-17, anti-TNFα, anti-IL-

6, anti-IL-12p40 and anti-IL-10 couple on beads for capture antibodies and biotinylated IL-1β, anti- IL-4, anti- IL-17, anti-TNFα, anti-IL-6, anti-IL-12p40 and anti-IL-10 for detection. Stretpavidin-conjugated PE was added to develop signals. The signals were detected by Bio-plex 200 systems (Biorad). Data were analyzed using Bio-Plex Manager™ software (Bio-Rad). * indicated statistical significance at $p<0.05$. The results represent mean ± SEM of each group (n=4).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0. Unpaired t-test ($p < 0.05$) was used when comparing two conditions.

CHAPTER IV

RESULTS

Plasticity of M(LPS) toward M(IC)

The plasticity between M(LPS) and M(IL-4) macrophages has been reported (4) and this function of macrophages plays an important role in progressions of several diseases (3). However, there was no report whether the reverse polarization from M(LPS) to M(IC) is possible. First, we tested the polarization of M(LPS) and M(IC) *in vitro*, following a protocol from the previous study using BMDMs (Fig 10) to confirm the phenotypes of M(LPS) and M(IC). In this protocol, BMDMs were primed with IFN-γ overnight before stimulation with LPS or LPS with IC. Next, we tested the re-polarization capacity of M(LPS) to become M(IC) as depicted in Fig 11A. In this study, we rested cells between the first and second stimuli for 2 days using BMDMs without IFN-γ priming following the previous study (62). We found that IFN-γ priming during M(LPS) polarization resulted in failure to re-polarize of M(LPS) to M(IC) (Fig 26). The results showed M(IC) that were polarized from resting macrophages or M(LPS) similarly increased IL-10 while decreased IL-12p70 at a comparable level (Figure 11B). Shorter resting period (6h or 20h) between the primary stimuli LPS and secondary stimuli LPS with IC, respectively, failed to yield cytokine profiles of M(IC) (Fig 27). This result suggested that with a resting period between the two stimuli of at least 2 days, M(LPS) has certain plasticity to be re-polarized from to M(IC) *in vitro*.

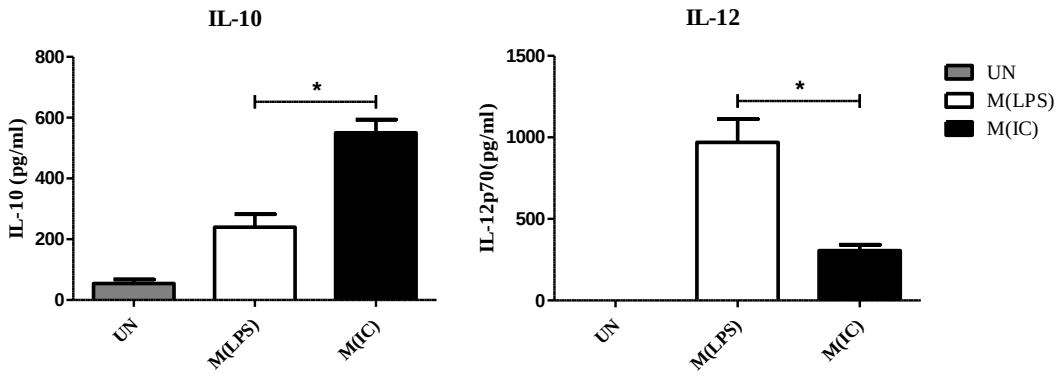


Figure 10 | Production of IL-10 and IL-12p70 in M(LPS) and M(IC)

BMDMs were primed with IFN-γ before stimulated with LPS or LPS/IC for 6h. IL-10 and IL-12p70 in the culture supernatant were measured using ELISA. The results indicated means ± SD of duplicate determined from 3 independent experiments. * indicated statistical significance at *p*-value < 0.05.

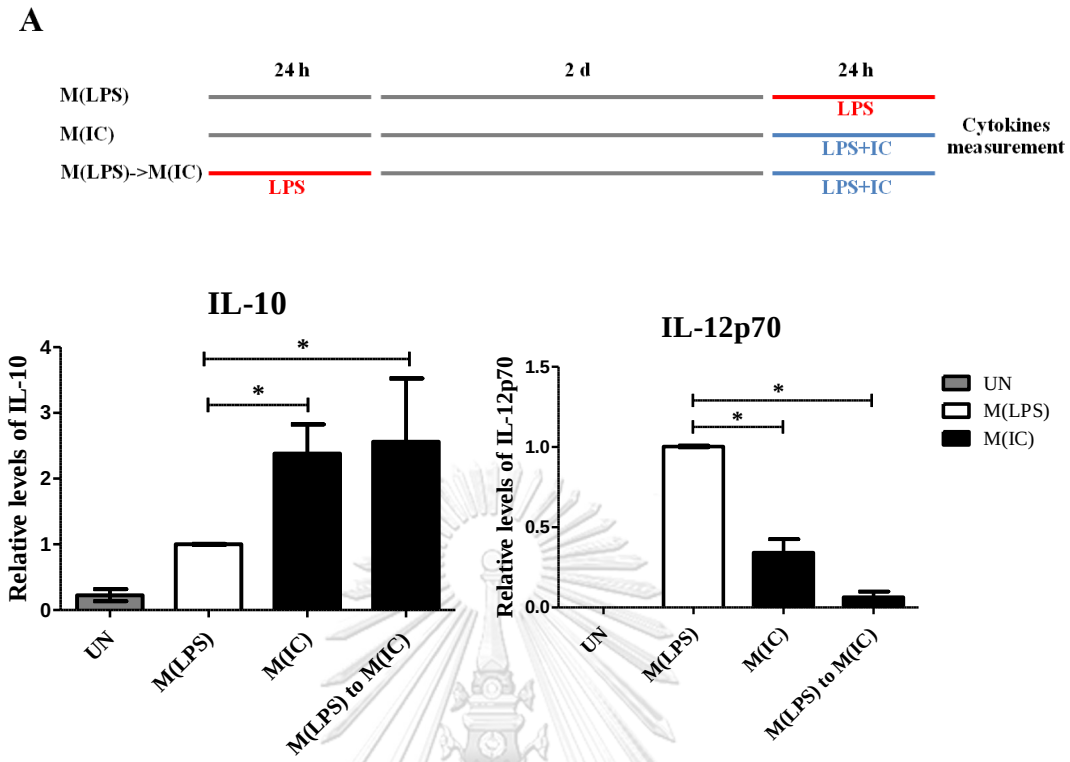


Figure 11 | Re-polarization of M(LPS) to M(IC)

(A) The protocol used to test the plasticity between M(LPS) and M(IC) is shown. (B) IL-10 and IL-12p70 in the culture supernatant harvested from cells treated as described in (A) were subjected to ELISA. The result is the relative level of IL-10 and IL-12p70 normalized to that of the control M(LPS) from means ± SD of triplicate determined of 3 independent experiments. * indicated statistical significance at *p*-value < 0.05.

Signaling pathways downstream of LPS/TLR4 and IC/FcγR in response to stimuli during polarization and re-polarization

From the results obtained above, we found that sufficient washout time of 2 days between LPS and LPS with IC was important for the observed plasticity. Therefore, we next investigated the early signaling pathway downstream of TLR4 and FcγR. We compared the downstream signaling pathway during re-polarization of short (2hours) and long (48hours) washout period as depicted in Fig 12. In the condition of 2 hours washout, markedly decreased phosphorylation of MAPK p38, SAPK/JNK, p44/42 and NF-κB p65 at 5, 15 and 30 min were observed in re-stimulation in response to LPS with IC when compare to M(IC) stimulated from naïve BMDMs. Phosphorylation of Akt was at a comparable level with the control (Fig 12A). In contrast, in the condition where plasticity toward M(IC) was obtained with the washout period of 2 days, the phosphorylation of MAPK p38, SAPK/JNK and p44/42 were similar to with those of M(IC) from naïve BMDMs. Interestingly, the phosphorylation of NF-κB p65 and Akt increased significantly in re-stimulated macrophages, compared to M(IC) from naïve BMDMs (Figure 12B). The results implied that the defects in early signaling pathways downstream of TLR4 and FcγR are associated with inability of M(LPS) to be re-polarized to M(IC) and the longer washout period between the two rounds of stimulation recovers this defect which allows M(LPS) to response to LPS and IC.

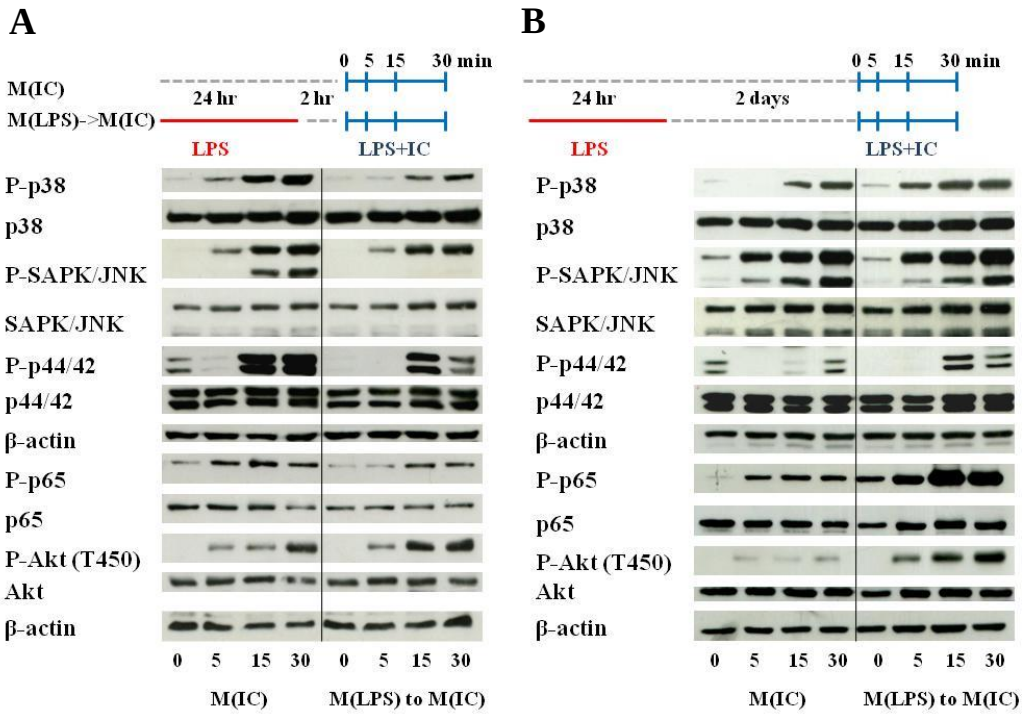


Figure 12 | Signaling downstream of TLR4 during the re-polarization

BMDMs were stimulated to M(LPS) and let rested in media for short washout time (2h) (A) or long washout time (48h) (B). After washout, cells were re-stimulated with LPS and IC, phosphorylation of MAPKs, NF-κB p65 and Akt were detected in cell lysates at 0, 5, 15 and 30 min after stimulation by Western blot. β-actin was used as a control. Representative data of the 2 independent experiments are shown.

Global H3K4me3 enrichment of M(IC) and M(LPS)

Besides the regulation of gene expression by the transcription factors that are intensively studied as short term regulatory mechanism, the epigenetic regulations including histone modifications have long term effect that also play important roles in regulating gene expression in activated macrophages (22). Because the duration of washout between the two stimuli is one of the key factors in the plasticity of re-polarization from M(LPS) to M(IC), we wondered whether the epigenetic modifications are different between M(LPS) and M(IC). Therefore, one of the epigenetic modifications of active histone marks, H3K4me3, were investigated by ChIP-seq and the profiles among IFN-γ primed macrophages (unstimulated macrophages), M(LPS) and M(IC) were compared. After mapping sequences to the references genome, Model-based analysis was used to identify significantly enriched H3K4me3 peaks with the *p*-value < 0.01. Circos plot was used to display global H3K4me3 enrichment among the three subsets of macrophages. The results revealed clear differences between the three subsets of activated macrophages (Fig 13). As shown in Fig 14, we found that the overall enrichment of H3K4me3 was higher in M(IC) than those of the unstimulated and M(LPS) and most H3K4me3 peaks that were enriched in M(LPS) overlapped with those in M(IC).

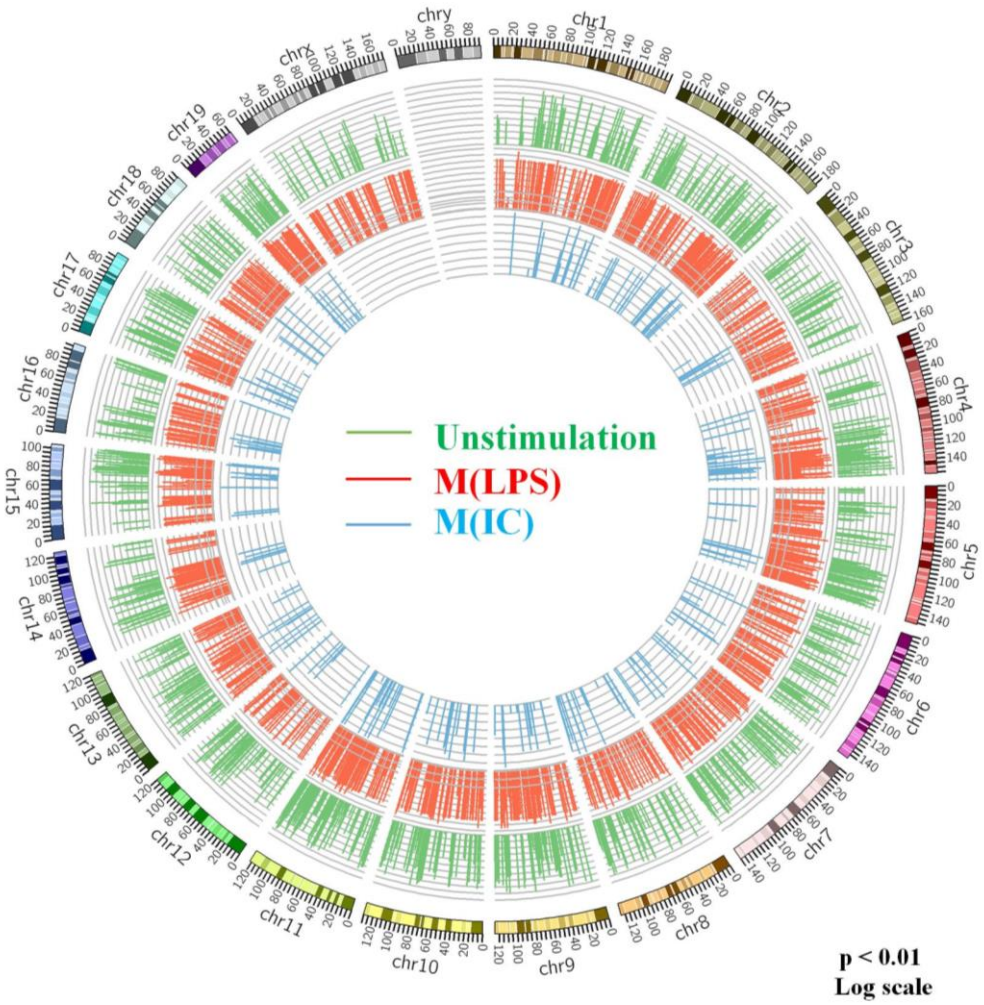


Figure 13 | Circos plot of the global enrichment of H3K4me3 in unstimulated macrophage, M(LPS) and M(IC)

BMDMs were primed with IFN-γ before stimulated with LPS with or without IC for 4 hours. Cells were harvested for ChIP using anti-H3K4me3 antibody. The ChIP DNA were subjected to next-generation sequencing. The Circos plot displayed genome-wide H3K4me3 enrichment in unstimulated macrophages (unstim), M(LPS) and M(IC). The position of log-transformed H3K4me3 enrichment of unstimulated macrophages (green circle), M(LPS) (red circle) and M(IC) (blue circle) were aligned according to the chromosome positions in the outer ring.

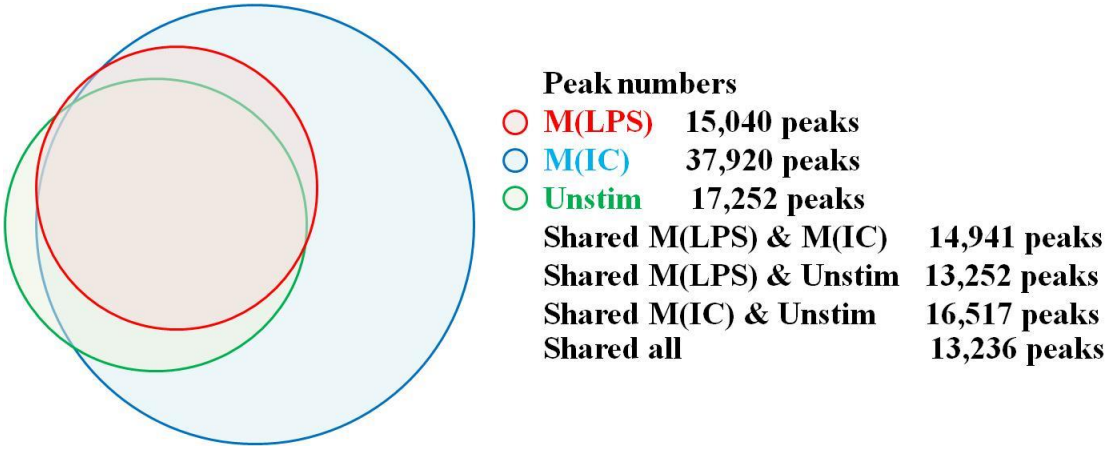


Figure 14 | Overlapping of H3K4me3 enrichments in unstimulated macrophage, M(LPS) and M(IC)

The total peaks of H3K4me3 enrichment with MACS 1.4 were used to compare the overlapping of H3K4me3 enrichment and showed peaks number among unstim (green), M(LPS) (red) and M(IC) (blue). The results are presented by Venn diagram.

Next, *Cis*-regulatory Element Annotation System (CEAS) was used to identify the distribution of ChIP regions in M(LPS) and M(IC). In both cases, the highest distributed peaks are enriched in the gene bodies, especially the promoter regions < 1000 bp upstream from TSSs (Fig 15). M(IC) showed more distributed peaks in the distal intergenic regions, the promoter regions (1000-2000 bp) while M(LPS) has more peaks distributed in the promoter regions (<1000 bp) and the coding exons. Taken together, these results revealed that M(IC) and M(LPS) displayed a similar pattern of global H3K4me3 enrichment profiles, but M(IC) showed higher H3K4me3 enrichment, especially in the distal intergenic and promoter regions.

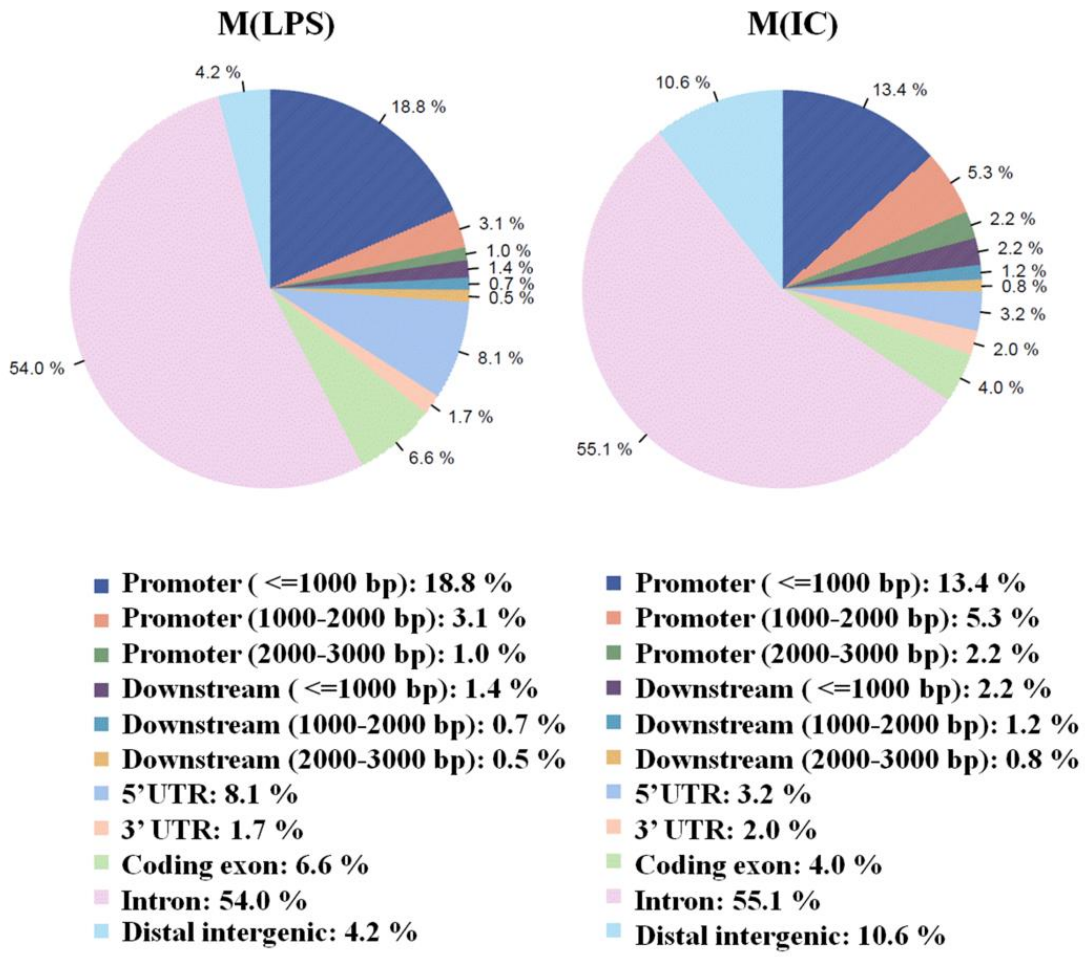
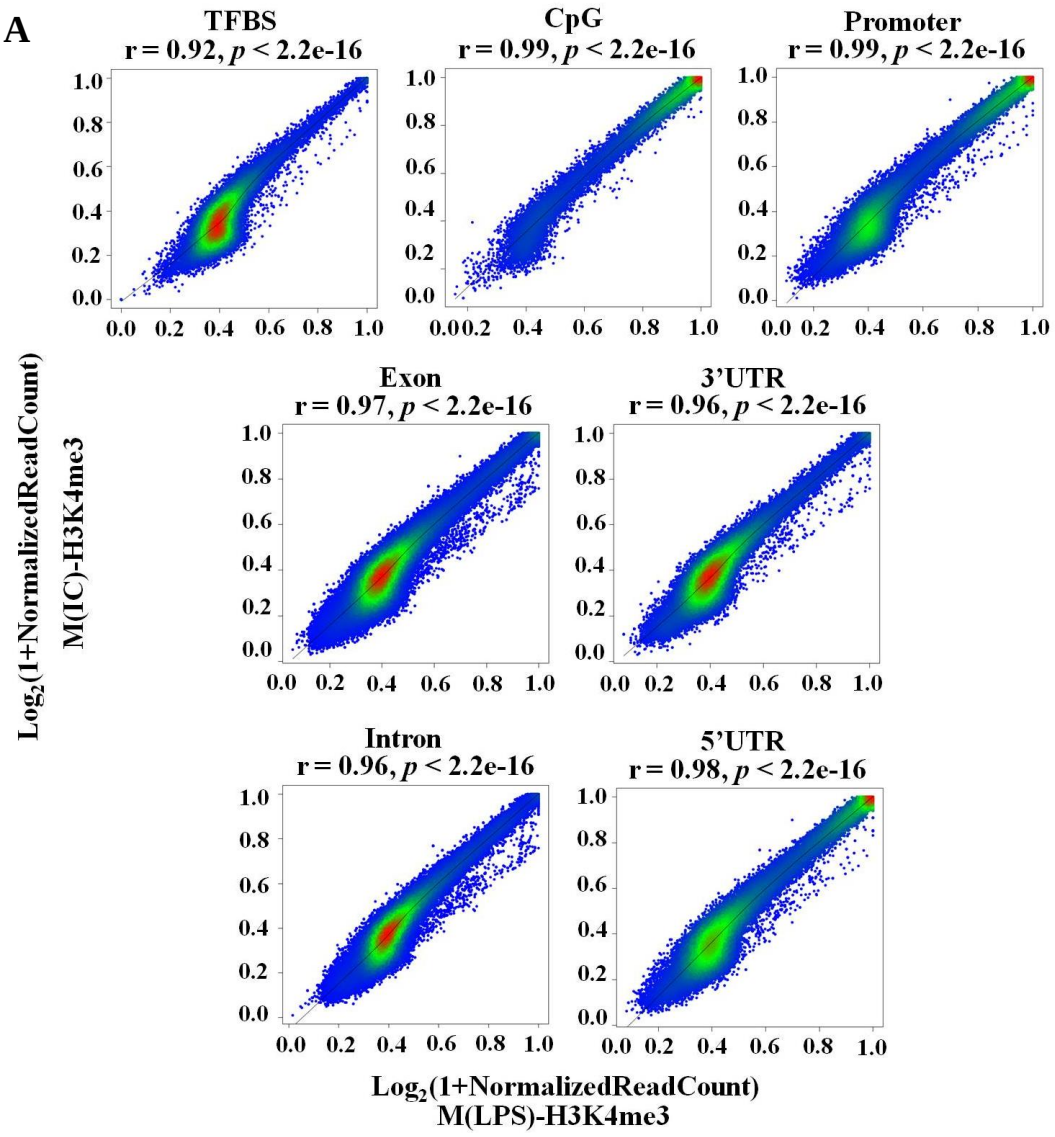


Figure 15 | Distributions of H3K4me3 enrichments in M(LPS) and M(IC)

CAES showed the distributing patterns of H3K4me3 enrichment between M(LPS) and M(IC). All ChIP-seq results were analyzed from combined RAW files of the 2 independent experiments.

The correlation of H3K4me3 peaks between M(LPS) and M(IC)

Next, we performed the correlation analysis at the genome-wide level using TCOR in EpiMINE. The datasets with two distinct correlation methods were analyzed using Pearson's correlation and principal component analysis (PCA). Pearson's correlation represented by the scatter plot demonstrated that the M(LPS) and M(IC) were highly correlated with Pearson's score of more than 0.90. The *cis*-regulatory elements include transcription factor binding sites (TFBS), CpG island and promoter regions and non-*cis*-regulatory elements regions are exon, 3'UTR, 5'UTR and intron (Fig 16A). More importantly, the PCA plot revealed that the two top principal components between M(LPS) and M(IC) in all *cis*-regulatory regions, exon, intron, 3'UTR and 5'UTR are distinct (Fig 16B). M(IC) displayed the different of two top principal components from M(LPS) and unstimulated macrophages in TFBS, CpG, exon and intron. While in promoter regions and 5'UTR, M(IC) showed the difference of two top principal components from M(LPS) only and similar enrichment level with unstimulated macrophages. These results implied that M(LPS) and M(IC) were epigenomically highly correlated but they have distinctive profiles in H3K4me3 enrichment. This pattern may result in the difference in the gene expression profile between these two phenotypically distinct effector macrophages.


A
TFBS
r = 0.92, p < 2.2e-16
CpG
r = 0.99, p < 2.2e-16
Promoter
r = 0.99, p < 2.2e-16
Exon
r = 0.97, p < 2.2e-16
3'UTR
r = 0.96, p < 2.2e-16
Intron
r = 0.96, p < 2.2e-16
5'UTR
r = 0.98, p < 2.2e-16
Log2(1+NormalizedReadCount)
M(IC)-H3K4me3
Log2(1+NormalizedReadCount)
M(LPS)-H3K4me3

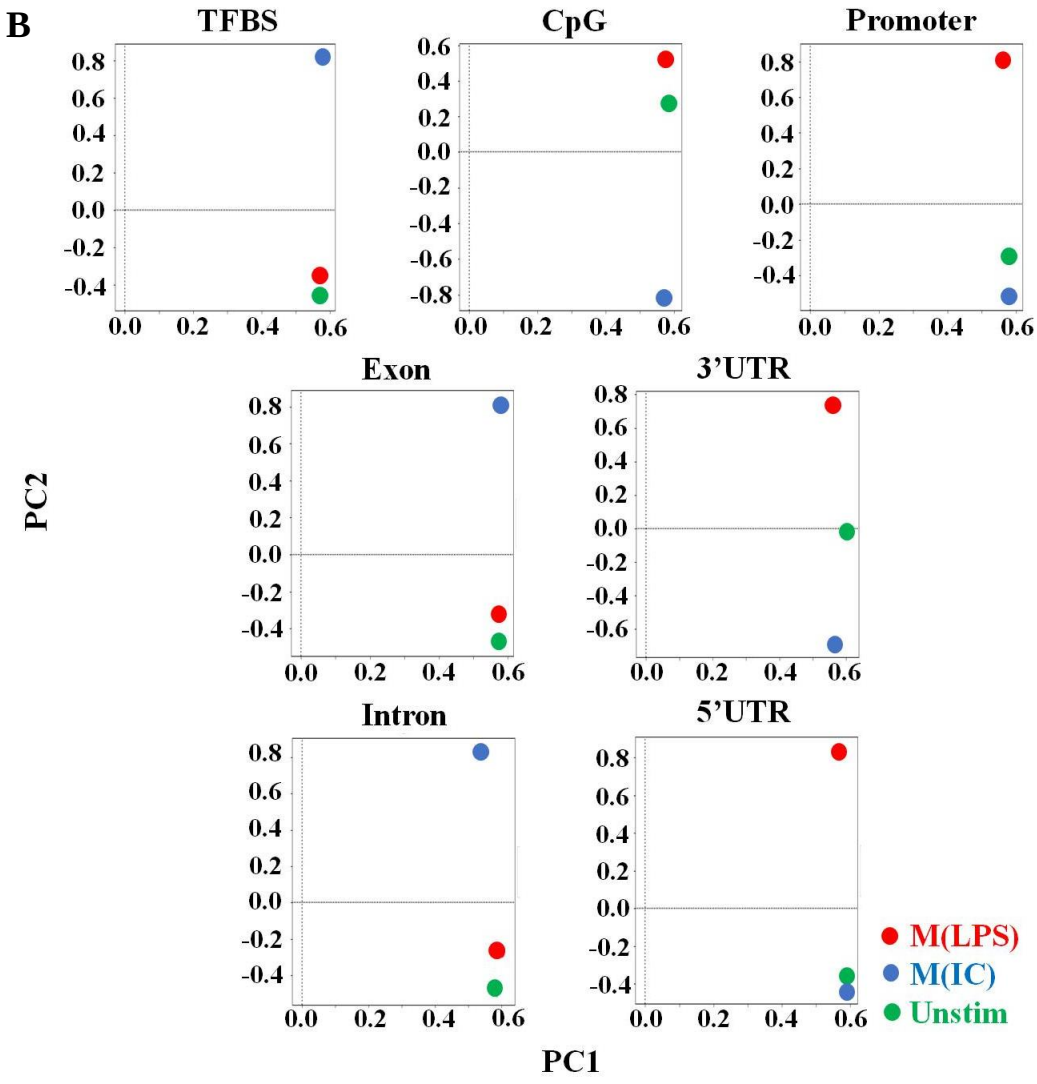


Figure 16 | The epigenomic correlation between M(LPS) and M(IC)

(A) A scatter plot from EpiMINE showed the epigenomic correlation between M(LPS) and M(IC) in the *cis*-regulatory elements and other regions. (B) The PCA plots showed the differences in the two top principal components between M(LPS) and M(IC).

H3K4me3 enrichment in the *cis*-regulatory elements of M(LPS) and M(IC)

Among the genome-wide enrichment, we focused on specific genomic regions of the *cis*-regulatory elements, including the promoters and the transcription factor binding sites (TFBS). Heatmap showed the H3K4me3 enrichment within the TSSs and 1 kb nearby TSSs (Fig 17A). M(IC) had higher signals of H3K4me3 enrichment when compared with M(LPS) and comparable signals when compared to unstimulated macrophages (Fig 17A). When the ChIP-seq peaks were analyzed by CEAS to obtain the average gene profiles of H3K4me3 within the TSSs and the 5 kb nearby, the average gene profiles of all three subsets of macrophages have similar patterns of H3K4me3 enrichment (Fig 17B). From previous results, M(LPS) showed the higher percentages of H3K4m3 enrichment distribution in the promoter regions than M(IC) (Fig 15). However, when the quantifies different datasets within specific region of interest (QIRI) was used to cluster H3K4me3 enrichment regions by k-mean, the results revealed the twenty clusters based on the level of presence to absence of H3K4me3 enrichment representing in the value from 0 to 1. It was found that at the promoter regions, the enrichment of H3K4me3 was higher in M(IC) than M(LPS) in all 20 clusters (Fig 18). Taken together, the results suggested that H3K4me3 enrichment of M(IC) in the promoter regions were higher than that of M(LPS) similar to the global H3K4me3 enrichment. The results suggested that FcγR signaling may be modulated signal from TLR4, resulting in the increasing of H3K4me3 enrichment to a similar level with unstimulated macrophages. These results also imply that M(IC) with the increasing enrichment of H3K4me3 in the promoter regions may have more active histone mark that is conducive to for gene transcription than M(LPS).

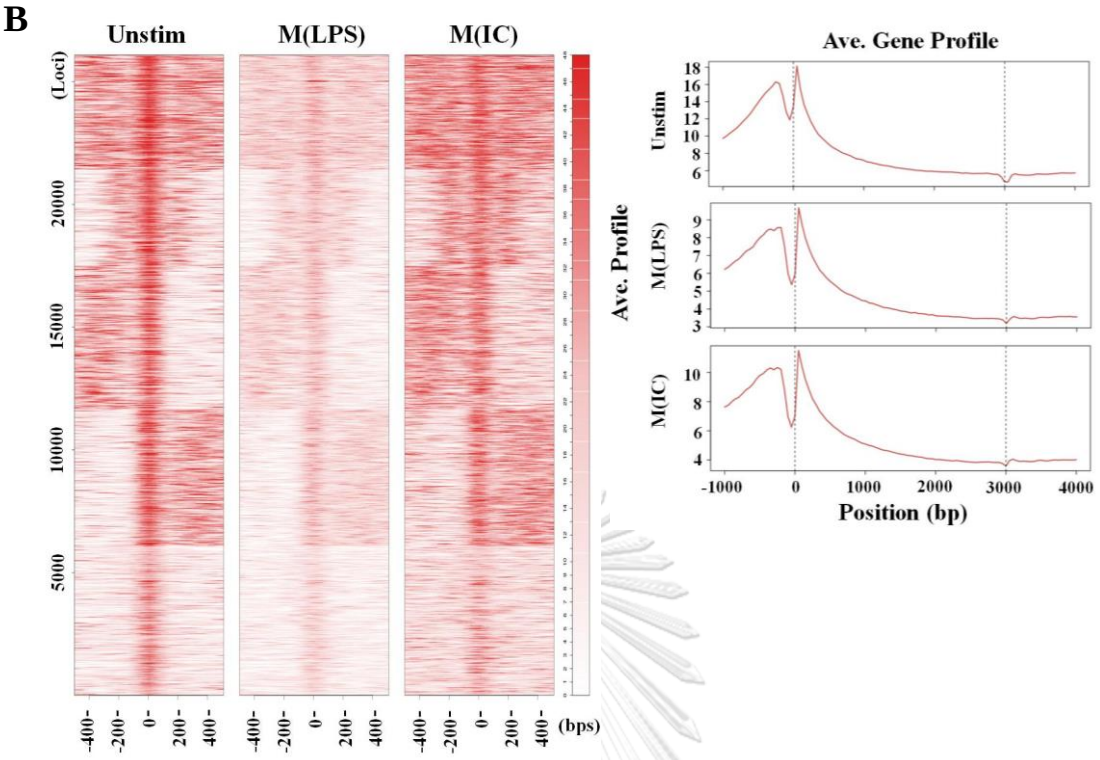


Figure 17 | The enrichment pattern of H3K4me3 in the cis-regulatory regions

(A) ChIP-seq density heatmap of H3K4me3 enrichment at the TSSs and the 1 kb region nearby. (B) ChIP-seq profiling of H3K4me3 enrichment over 5 kb window around TSSs.

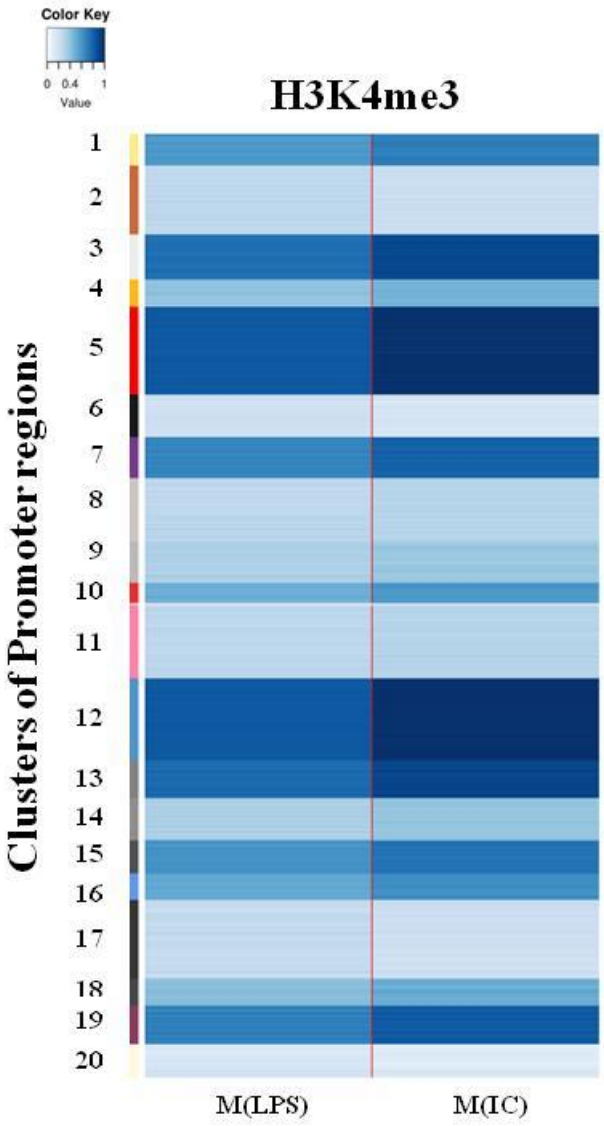


Figure 18 | Quantification of H3K4me3 enrichment within the promoter regions

QIRI was used for quantification of H3K4me3 enrichment between M(LPS) and M(IC) within the promoter regions (1kb) using k-mean clustering to twenty clusters. The different of H3K4me3 enrichment between M(LPS) and M(IC) were compared and showed in the level of presence to absence of H3K4me3 enrichment representing in the value from 0 to 1.

H3K4me3 enrichment in the regulatory regions of individual genes uniquely associated with M(IC)

To link the H3K4me3 profiles with the mRNA transcription of the genes, Integrative Genomics Viewer (IGV), was used to visualize specifically targeted locus. We investigated H3K4me3 enrichment in all three subsets of macrophages of the uniquely upregulated genes i.e. *Il10, Cxcl1, Csf3* and *Il33* and uniquely downregulated genes i.e. *Il12b* and *Il6* as reported by RNA-seq (9). The result showed the increased H3K4me3 enrichment in upregulated genes *Il10, Cxcl1, Csf3, Il33* in M(IC) when compared with M(LPS) and unstimulated macrophages (Fig 19A). In contrast, the H3K4me3 enrichment in downregulated genes *Il12b* and *Il6* were not different among the three subsets of macrophages (Fig 19B). The quantification H3K4me enrichment by QIRI showed most of the genes that are reported induced during LPS with IC stimulation as described by Fleming et al. from RNA-seq data (9) increased the enrichment when compared to those in M(LPS) (Fig 20). Overall, the H3K4me3 enrichment correlated well with the uniquely upregulated genes in M(IC), suggesting that active histone mark H3K4me3 plays active roles in regulating the expression of signature genes in M(IC) such as IL-10.

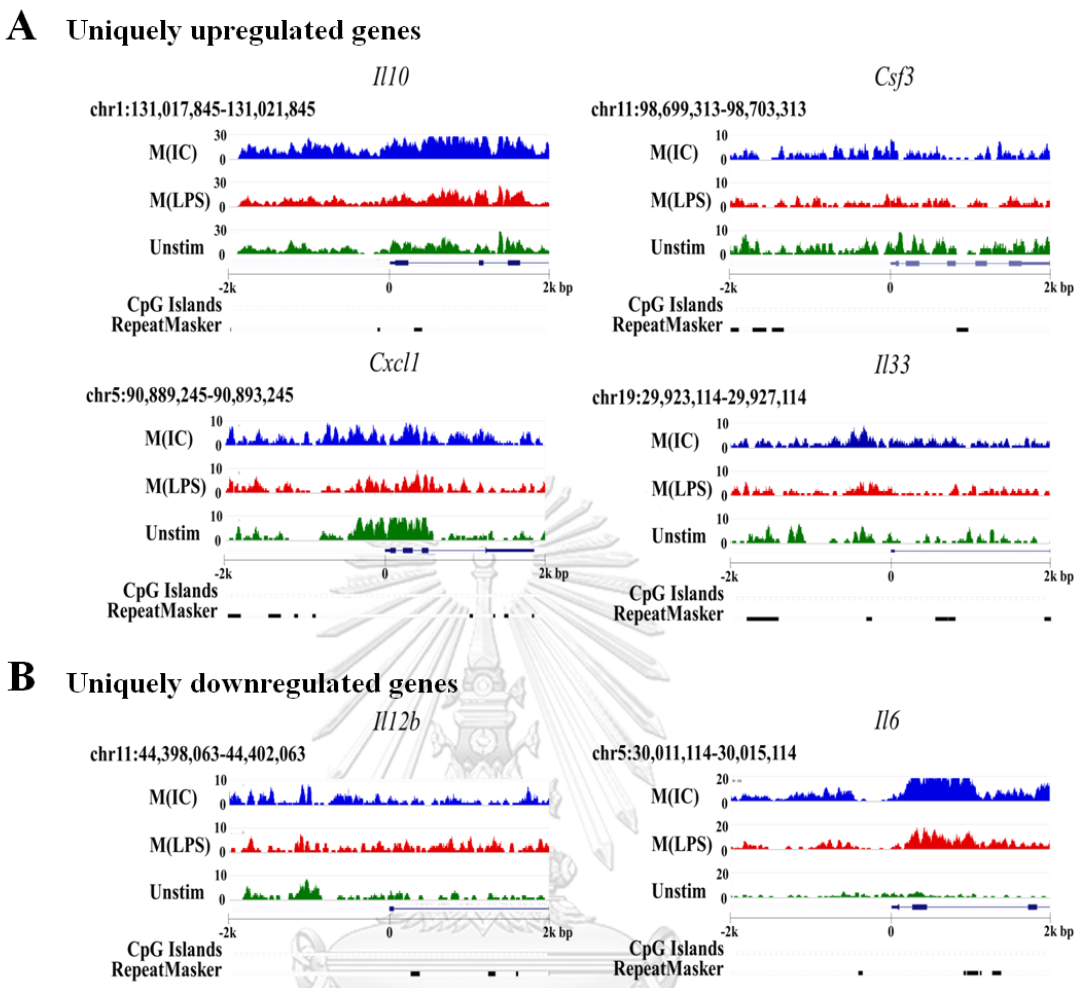


Figure 19 | Enrichment of H3K4me3 in the target locus

IGV was used to visualized H3K4me3 enriched peaks in M(IC)-related loci from RNA-seq data by Fleming et al. (9) of uniquely upregulated genes (A) and uniquely downregulated genes (B). The figures represent peaks of unstim (green), M(LPS) (red) and M(IC) (blue) in target locus at TSSs and 4 kb nearby. Promoter regions, CpG island and repeat regions are regions that may contain TFBS and functional motifs in the sequences.

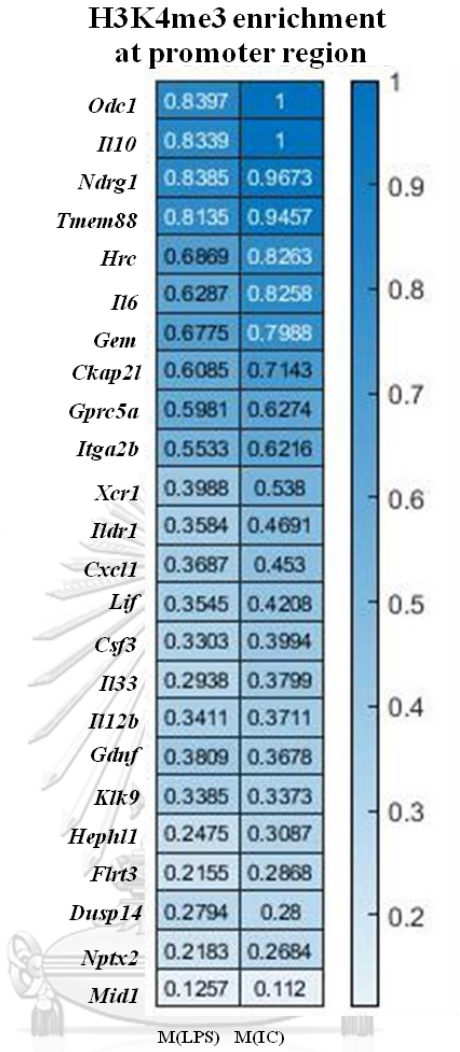


Figure 20 | Quantification of H3K4me3 enrichment related genes

Quantification of H3K4me3 enrichment in the promoter regions of the target genes between M(LPS) and M(IC). The level of presence to absence of H3K4me3 enrichment are representing in the value from 0 to 1.

Prediction of potential associated regulators/transcription factors enrichment motifs in M(IC)

MEME-ChIP was used to identify novel enrichment motifs and the possible associated regulators or transcription factors that showed significant increased H3K4me3 in M(LPS) and M(IC). The results showed the top 10 of motifs that were highly enriched in M(LPS) and M(IC) (Fig 21). M(IC) and M(LPS) have some of overlapping known motifs, including Nr5a2, KLF5, Klf4, SP1, EGR1 and YPRo22C (Fig 21). However, M(IC) had some unique unknown and known motifs such as stat4 and STAT1 that were highly enriched (Fig 21). These motifs may be the novel motifs for transcription factors or binding site for epigenetic modifiers that are important in M(IC) polarization.

M(LPS)

Motif	Logo	RC Logo	E-value	Known/Similar motifs
1.GAGRCAGR			8.1e-051	
2.CKGGRA			1.3e-048	
3.RGAAA			7.3e-038	AGL27, FLC, AZF1, Bcl6
4.GGKCTACA			3.0e-033	
5.GAGTTCRA			5.8e-032	Nr5a2
6.CCCCWYCC			2.9e-027	KLF5, Klf4, SP1, EGR1, Klf1, ZNF740,RGM1, YPR022C, SP2
7.RCCTTTAA			9.7e-025	
8.RCCACCAY			1.5e-023	GLI2
9.AAAAYMAA			4.2e-022	Sox3, AZF1
10.GTGKGTR			3.7e-021	daf-12

M(IC)

Motif	Logo	RC Logo	E-value	Known/Similar motifs
1.ARTYCCAG			6.1e-081	
2.GAGGMAGR			3.7e-065	
3.TVTGTR			1.9e-047	
4.ARGCCAGC			5.6e-047	Nr5a2
5.CCMCDCCC			1.6e-046	KLF5, Klf4, SP1-2, EGR1, Klf16, YPR022C, TDA9
6.AACTCAGR			1.4e-035	YLR278C
7.TTTAWW			5.9e-031	AHL20
8.RRGAAA			6.8e-028	Stat4, STAT1, Stat92E
9.CCAKCHC			3.9e-025	
10.CACGB			1.4e-021	usp, Pax2

Figure 21 | The novel enrichment motifs and possible associated regulators/transcription factors in M(IC)

MEME-ChIP was used to identify the novel enrichment motifs and possible associated regulators/transcription factors from significantly enriched peaks ($p < 0.01$) after peak calling. The top 10 motifs that were highly enriched in M(LPS) and M(IC) are shown.

KEGG Pathway analysis of gene with differential H3K4me3 enrichment in M(IC)

Gene Ontology analysis using KEGG pathway was performed to investigate the possible regulatory molecules/signaling pathways revealed by ChIP-seq data in M(IC) and M(LPS). We used genes that showed uniquely H3K4me3 enriched profile in M(IC) or M(LPS) and filtered by the KEGG pathway using the immune system, signal transduction and signaling molecule to obtain the interaction profiling data. We found 10 significantly enriched pathways in M(IC) (Fig 22). Among these pathways, cytokine-cytokine receptor interaction was the top highly enriched in M(IC). Furthermore, M(IC) also showed significant enrichment of the cell adhesion molecules, RAP1 and cAMP signaling pathways. For M(LPS) enriched pathways, there was no significantly enriched pathway that uniquely in M(LPS) (Fig 22). This result suggested that increasing of H3K4me3 enrichment may regulate gene expression by inducing genes of downstream signaling pathways that are crucial for M(IC) polarization while M(LPS) may use other epigenetic regulation for its polarization related genes.

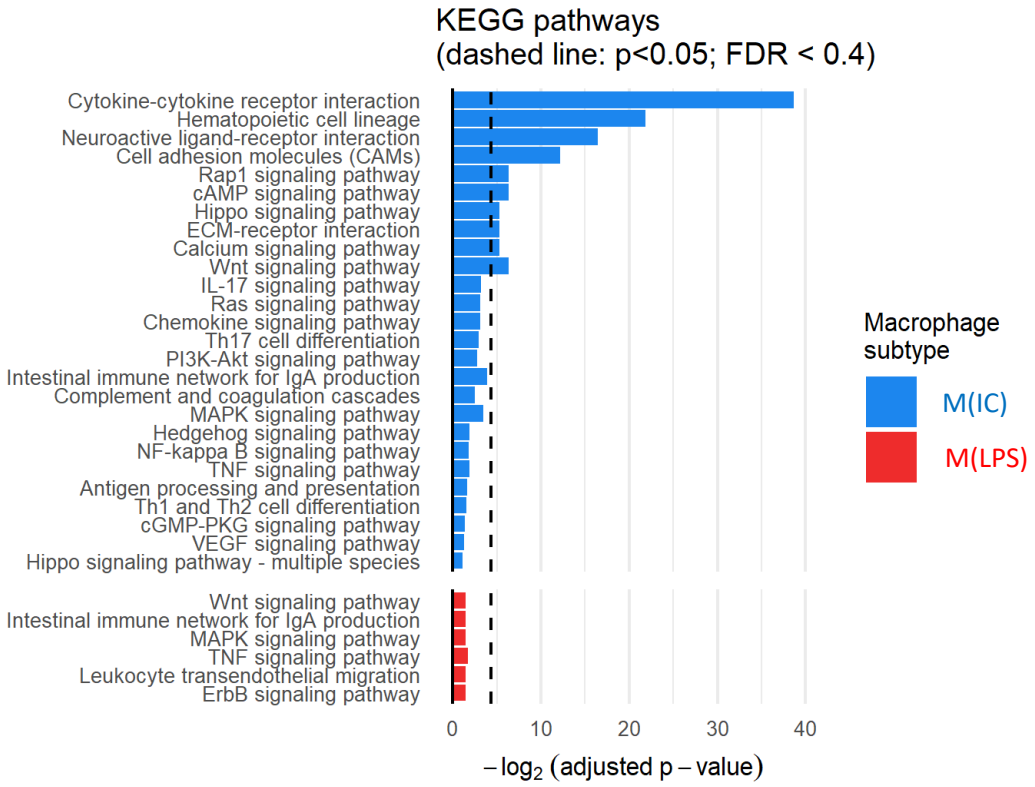


Figure 22 | KEGG pathway analysis of the differentially H3K4me3 enrichment genes in M(IC) and M(LPS)

Gene Ontology analysis using KEGG pathway analysis displayed the significant pathways with differential H3K4me3 enrichment genes in M(IC) and M(LPS). The bar plot showed the ranking of pathways correlated to Immune system, Signal transduction and Signaling molecule (*p*-value <0.05) in M(IC) and M(LPS).

Effect of H3K4me3 demethylase inhibition on M(LPS) plasticity

To investigate the role of H3K4me3 in M(LPS) plasticity toward M(IC), we tested the effect of H3K4me3 demethylase inhibition on M(LPS) plasticity. We treated BMDMs with H3K4me3 demethylase inhibitor which increased H3K4me3 in 2h resting time before re-polarization M(LPS). We expected that the increase of H3K4me3 may help to modulate M(LPS) plasticity toward M(IC) by increase IL-10 production and re-polarization of M(LPS) to M(IC) similar to that of the 48h resting time without inhibitor. The results showed that repolarized M(LPS) treated with IOX-1 (histone H3 demethylase inhibitor) or JIB-04 (H3K4me3 demethylase inhibitor) did not increase IL-10 production when compared to M(LPS) control (Fig 23). This results suggested that the increase in H3K4me3 alone may not be sufficient in modulating M(LPS) plasticity in 2h resting time before re-polarization.

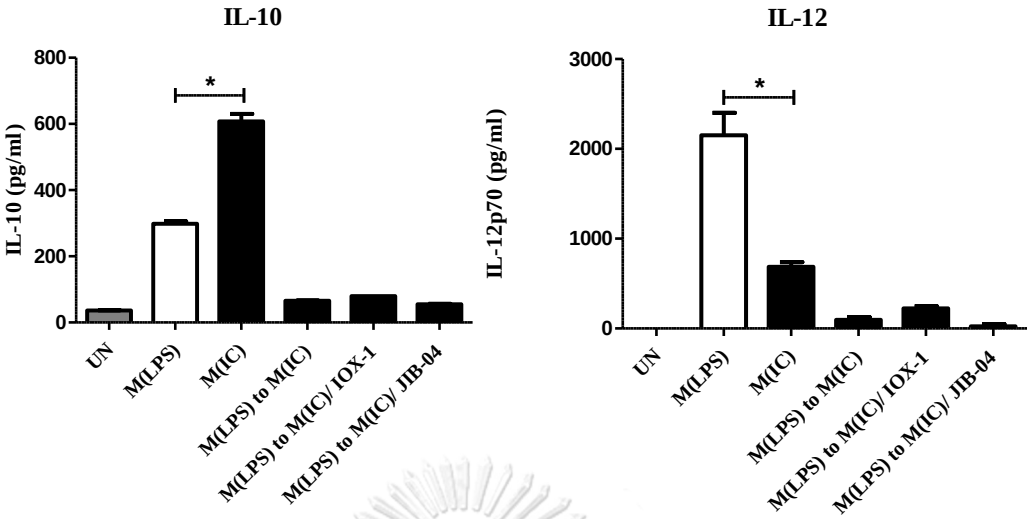


Figure 23 | The effect of H3K4me3 demethylase inhibitor on re-polarization of M(LPS) to M(IC)

(A) The protocol used to study the effect of H3K4me3 demethylase on the plasticity of M(LPS) to M(IC) is shown. (B) IL-10 and IL-12p70 in the culture supernatant harvested from cells treated as described in (A) were subjected to ELISA. The results indicated means ± SD of triplicate determined from 1 of 3 independent experiments. * indicated statistical significance at *p*-value < 0.05.

The effect of adoptive transfer of M(IC) on systemic cytokines profiles in mouse model of LPS-induced endotoxemia

Previously, adoptive transfer of M(IC) was reported to rescue sepsis mouse model (9). To evaluate the impact of M(IC) *in vivo*, we tested the effect of adoptive transfer of M(IC) on the systemic cytokine profiles in LPS-induce endotoxemia model using protocol depicted in Fig 24. Production of pro-inflammatory cytokines IL-1β and IL-12p70 were significantly decreased at 6 hours after LPS challenge in mice receiving an adoptive transfer of M(IC), compared to those receiving unstimulated macrophages (Fig 25). In contrast, the level of other inflammatory cytokines IL-6, TNF- α and IL-17 were not significantly different between the two groups (Fig 25). Furthermore, there was no difference for anti-inflammatory cytokine IL-10 and Th2 cytokine IL-4 between the two groups (Fig 25). The cytokines profiles revealed that the adoptive transfer of M(IC) in mice with LPS-induced endotoxemia before LPS administration has systemic impact on some pro-inflammatory cytokines but with no detectable influence on IL-10.

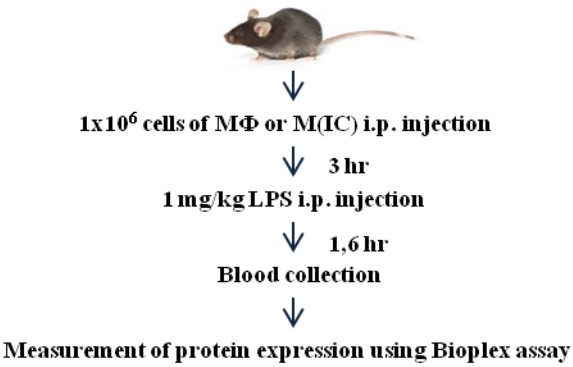


Figure 24 | Experimental set up to investigate the therapeutic application of M(IC) in endotoxemia mouse model

The protocol used for adoptive transfer of M(IC) in endotoxemia mouse model. Female mice (n=4/group) were used.

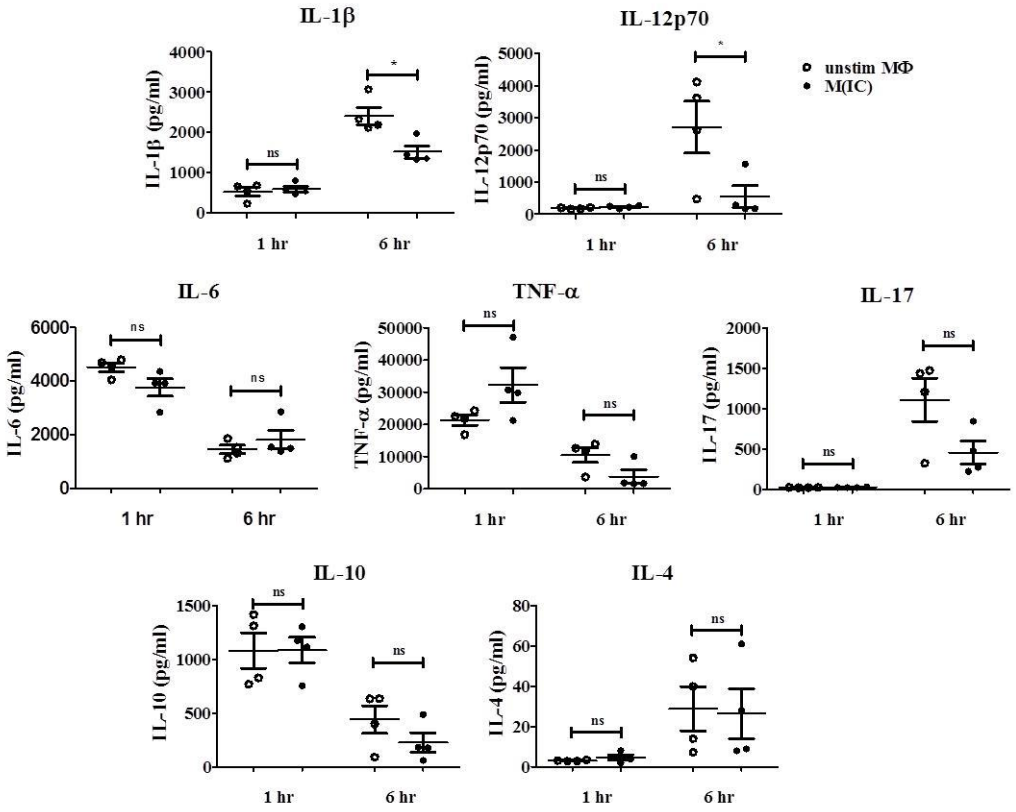


Figure 25 | The cytokines profile of endotoxemia mice adoptive transfer with M(IC)

Blood serum at 1 and 6 hours after LPS challenged from mice with adoptive transfer of unstimulated macrophages or M(IC) were subjected to Bio-plex cytokine assay for IL-6, IL-12p70, IL-1β, TNF-α, IL-17, IL-10 and IL-4. * indicated statistical significance at *p*-value < 0.05. The results represent mean ±SEM of each group (n=4).

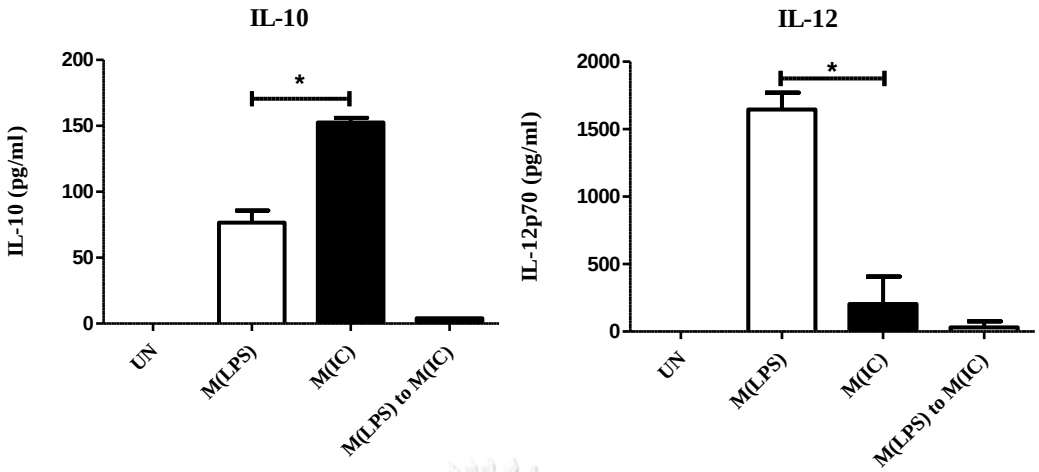


Figure 26 | Test IFN-γ priming for re-polarization M(LPS) to M(IC)

BMDMs were priming with IFN-γ before primary stimulated with LPS for 4h, washed and rested in media for 2h before second stimulated with LPS/IC for 4h. IL-10 and IL-12p70 in the culture supernatant were measured using ELISA. The results indicated means ± SD of triplicate determined from 1 of 3 independent experiments. * indicated statistical significance at *p*-value < 0.05.

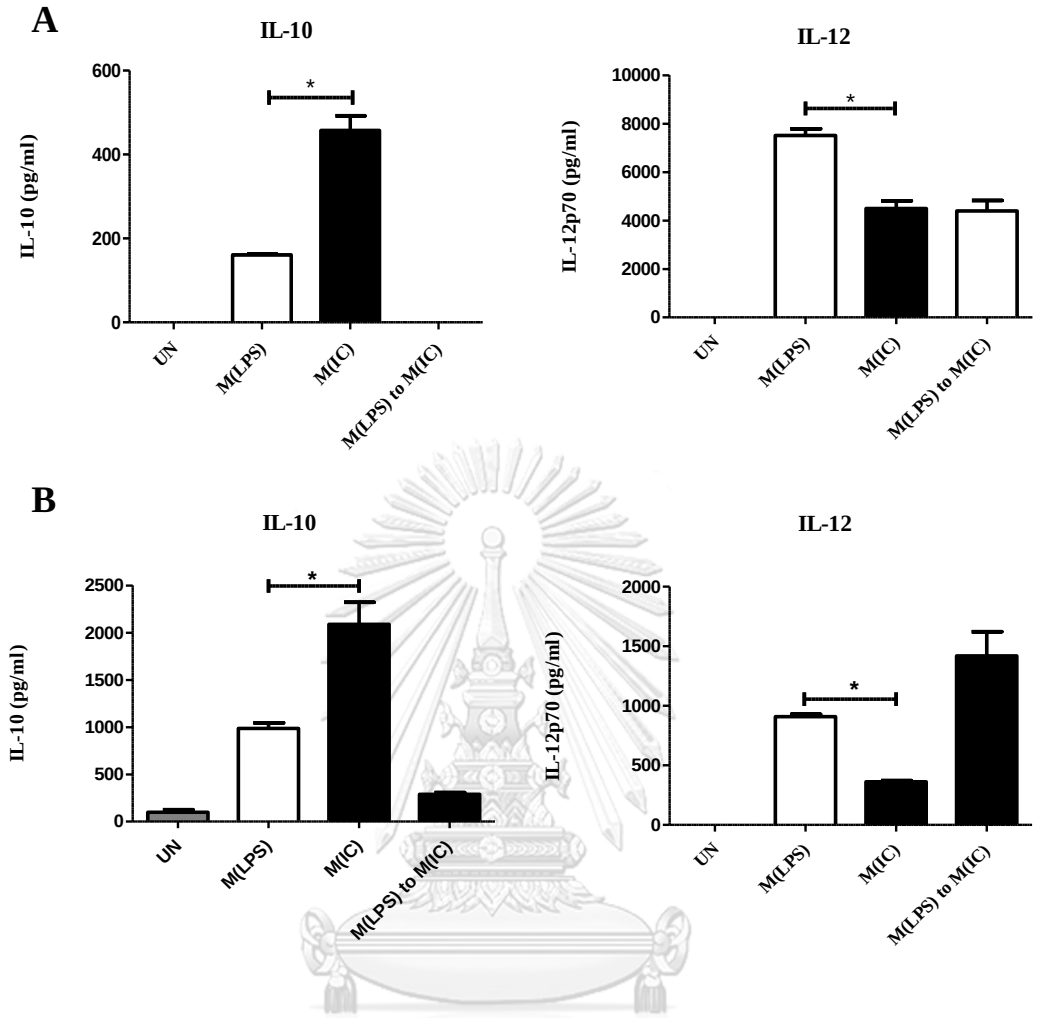


Figure 27 | Shorter washout period fail to re-polarization M(LPS) to become M(IC)

BMDMs were primarily stimulated with LPS for 24h, washed and rested in media for 6h (A) or 20h (B) before second stimulated with LPS/IC for 24h. IL-10 and IL-12p70 in the culture supernatant were measured using ELISA. The results indicated means ± SD of triplicate determined from 1 of 3 independent experiments. * indicated statistical significance at *p*-value < 0.05, ns indicated not significantly different.

CHAPTER V

DISCUSSION

Macrophages have the diverse population depending on development origins. Its functional diversity and plasticity are their important characteristics of macrophage that play an important role in homeostasis and various diseases (3, 24). LPS in the presence of immune complex stimulated macrophage to become regulatory effector macrophages that produce high level of anti-inflammatory cytokine IL-10 while reduces pro-inflammatory IL-12 production (63). Extensive gene expression profiles of M(IC) was reported and show they are a distinct subset of activated macrophages (9). It is well known that macrophages have functional plasticity that allows them to switch phenotypes between M1 or M(LPS) and M2 or M(IL-4). Many studies reported on the association of macrophages and diseases pathology and progression (24). However, it remained unclear whether M(LPS) have functional plasticity that allows it to be re-activated to become M(IC). This is an important question in light of the therapeutic implications of M(IC) in septicemia and autoimmune diseases such as multiple sclerosis (9, 10).

In this study, we demonstrated that M(IC) had functional plasticity that can switch from M(LPS) to M(IC) *in vitro*. However, certain length of resting time is important for this plasticity. Moreover, we found that the MAPK and NF-κB signaling pathways were reduced when short resting time (2h) was applied while the signaling recovered in the long resting time before second stimuli. It is possible that after first stimulation macrophages have some epigenetic modification leading to the decreased downstream signaling pathway-associated with M(IC). We also found that IFN-γ primed

macrophages did not response to the second even when longer resting time was applied. It is possible that IFN-γ signaling also modified some epigenetics leading to cellular unresponsive to TLR4/FcγR stimulation in the second stimulation. Upon LPS stimulation, TLR4 may be internalization that can result in reduced immediate signaling upon restimulation in short resting period.

Previous study demonstrated that ERK activation and the histone modification by phosphorylation of serine 10 on H3 played a key role in regulating IL-10 expression in M(IC) (22). In this study, we focused on H3K4me3 as an active histone mark, because it is the most dynamic epigenetic modifications in activated macrophage found at the gene promoters (64). H3K4me3 are histone mark that associate with actively transcribed genes (65). By ChIP-seq analysis, we found that M(IC) had high H3K4me3 enrichment globally and in all *cis*-regulatory regions. Moreover, the quantification study revealed that the H3K4me3 enrichment in promoter regions showed highly H3K4me3 enrichment in M(IC) in all cluster. It was correlated with the type of this modification that usually found in the promoter regions. It is possible that signal from FcγR including Syk, Ras and PI3K (29) modulated histone modification H3K4me3 resulting in the increasing of H3K4me3 when compared to macrophage receiving TLR signal alone.

Interestingly, the results also showed the increasing of H3K4me3 enrichment in the promoter regions of most of interested target loci that are uniquely upregulated in M(IC), i.e. *Il10*, *Cxcl1*, *Csf3* and *Il33*. Our study revealed for the first time that H3K4me3 in M(IC) is another epigenetic modification macrophages use to regulate IL-10 expression. Besides *Il10*, the promoters of *Odc1*, *Ndgr1*and *Tmem88* showed the

marked difference between M(LPS) and M(IC). *Odc1* encodes ornithine decarboxylase, a rate-limiting enzyme of the polyamine biosynthesis pathway. Ornithine decarboxylase is reported in macrophages to regulate M1 and specific deletion of this gene in macrophages results in hyperactivation of M1 and exacerbates colitis (66). Furthermore, ornithine decarboxylase modifies histone that impinges upon M1 gene expression (67). Our results imply that M(IC) may regulate *Odc1* expression by increasing H3K4me3 in the promoter region. Ndgr1 encodes N-myc downstream regulated 1, a protein member of the NDRG family. Interestingly, NDGR1 KO mice exhibited impaired differentiation of M1/M2-type macrophage differentiation and its expression was found in tumor-infiltrating macrophages of renal cancer (68, 69). *Tmem88* encodes Transmembrane Protein 88 (Tmem88), a protein that regulates Wnt/beta-catenin signaling pathway, one of the pathway that highly H3K4me3 enrichment in M(IC). Moreover, previous study reported that Tmem88 is regulating pro-inflammatory cytokines secretion through JNK/p38 MAPK signaling pathway (70).

For the downregulated genes in M(IC), *Il6* and *Il12b* showed no significant difference in H3K4me3 enrichment between M(LPS) and M(IC). This result suggested that the regulation of downregulated genes in M(IC) may have other histone modification that overrides H3K4me3 play dominant roles in the regulation of downregulated genes expression.

Epigenetics included variety of regulatory mechanisms including many types of histone modifications both permissive and repressive, DNA methylation, non-coding RNA mediated regulations. In fact, many types of epigenetic modification were reported during macrophage activation and function (18, 71). It is possible that several

epigenetic modifications coordinating play a role in regulation of gene expression. Therefore, we cannot rule out that other epigenetic regulations may also play important roles in M(IC) and its plasticity.

For the epigenomic correlation, TCOR in EPIMINE was used to analyze the correlation between M(LPS) and M(IC). To our surprise, these two phenotypes of macrophages had positive correlation of H3K4me3 enrichment in all *cis*-regulatory elements and in other regions, even though the PCA plot showed the difference of two top principal components of enrichment between M(LPS) and M(IC). The results suggested that these two phenotypes of macrophage are closely related. However, they have some different in H3K4me3 enrichment that may one reason of the different in the phenotype during the activation.

We further investigated the possible regulatory molecules/signaling pathway using MEME ChIP and KEGG pathway analysis. MEME-ChIP result displayed some of unique unknown motifs that were highly enriched only in M(IC). These unknown motifs may be the novel motifs for transcription factors or epigenetic modifiers binding that are important in M(IC) polarization. Surprisingly, unique known motifs that are enriched in M(IC) such as STAT4 and STAT1 positively correlated with IL-12 production and pro-inflammatory response in macrophages in contrast with their phenotype (72, 73). The increasing H3K4me3 enrichment in STAT1 and STAT4 maybe because these two transcription factors are IFN-γ dependent that can increase in IFN-γ primed macrophages.

H3K4me3 enrichment was found in regulatory regions of genes associated with cytokine-cytokine interaction, hematopoietic cell lineage, cell adhesion molecules, Wnt

signaling pathway and RAP1 signaling pathway by KEGG. All of these pathways were demonstrated to be involved in macrophage function and activation. RAP1 signaling regulates downstream signaling of MAPK and PI3K/Akt (74) that crucial for M(IC) polarization and plasticity. Wnt/β-catenin pathway is linked to alternatively activated macrophage that contributes to kidney fibrosis (75, 76). Therefore, trimethylation of H3K4 may be a key epigenetic mechanism that macrophages use to regulate gene expression during macrophage activation.

Finally, the using of M(IC) for therapeutically approach were tested, Previous study was reported that the adoptive transfer of M(IC) help to increased survival rate in LPS-induced endotoxemia mouse model when compared to mice adoptive transfer with unstimulated macrophage and other types of regulatory macrophages, but the impact on the cytokine profiles has not been explored (9). Therefore, we investigated the cytokines profiles in blood serum of mice receiving an adoptive transfer of M(IC) in sepsis mouse model. We found that mice adoptive transfer with M(IC) specifically decreased IL-1β and IL-12p70 level in blood serum when compared to mice adoptive transfer with unstimulated macrophages (Fig 25). However, the IL-10 level showed no significant difference between the two groups (Fig 25). In an EAE model, adoptive transfer of M(IC) reduced the disease severity EAE and splenocytes from EAE immunized mice receiving M(IC) produced substantially greater of IL-10 and IL-4 upon re-stimulation (10). Currently, it remains unknown how adoptive transfer of M(IC) decreased IL-1β and IL-12p70 level in blood serum. It is possible that M(IC) through IL-10 directly modulate cytokine production resulting in the decreasing of pro-inflammatory cytokines production. Alternatively, M(IC) may indirectly affect IL-1β and IL-12p70 production by other mechanisms. One of possibility maybe, M(IC)

enhances expression of MHC class II and co-stimulatory molecule CD86 (77) that may increase the function of antigen presentator and promoting regulatory T cells functions (78, 79), resulting in the inhibitory of pro-inflammatory cytokines production in macrophages and other cells.

Furthermore, the reason that IL-10 level showed no difference between mice adoptive transfer with M(IC) and unstimulated macrophages also remains unclear. It is possible that at that late time (more than 1 hour after LPS challenge), M(IC) that have functional plasticity received second stimuli from LPS in microenvironment and changed phenotype to become M(LPS), resulting in low IL-10 production and did not significantly different when compared to adoptive transfer with unstimulated macrophages. Therefore, manipulation of M(IC) polarization and plasticity still needs further research to make it able to be use for therapeutic application in the future.

Taken together, we demonstrated that M(LPS) has functional plasticity that can re-polarized to M(IC) at least *in vitro*. This event may be shad a new light on the further study about the manipulate cell phenotype for therapeutic application in inflammatory-related diseases. The resting time before receiving second stimulation is a key for recovery of the activation of downstream signaling pathways which are important for M(IC) polarization and plasticity. We also showed that active histone mark H3K4me3 is higher in M(IC) than that in M(LPS) in the genome-wide, promoter regions and M(IC)-related genes. The significantly enriched pathways in M(IC) are associated with cytokines and signaling downstream that regulated gene expression in M(IC). Moreover, we investigated the therapeutic application of M(IC) that decreased the production of pro-inflammatory cytokines in sepsis mouse model. Therefore, the

manipulation of the epigenetic regulation by H3K4me3 may help manipulate M(IC) polarization and its plasticity that can be therapeutically applied in the future.

CHAPTER VI

CONCLUSIONS

In conclusion, M(IC) and M(LPS) has functional plasticity *in vitro*. In the condition of short resting time, M(IC) that re-polarized from M(LPS), markedly decreased the phosphorylation of MAPK and NF-κB when compare to M(IC) stimulated from naïve BMDMs. However, the increasing of resting time between the two opposing stimuli is a key for recovery of the phosphorylation of importance downstream signaling pathways. We also showed that active histone mark H3K4me3 increased in M(IC) more than M(LPS) in both globally and M(IC) associated genes. M(LPS) and M(IC) were highly correlated with the Pearson score of more than 0.90. The significantly enriched pathways in M(IC) are associated with cytokines and signaling downstream that regulated gene expression in M(IC). Moreover, we investigated the therapeutic application of M(IC) that decreased the production of pro-inflammatory cytokines in sepsis mouse model. Therefore, our findings may help to better understanding the manipulation of the epigenetic regulation by H3K4me3 that may help manipulate M(IC) polarization and its plasticity that can be therapeutically applied in the future.

APPENDIX

LIST OF PREPARING REAGENTS

1) DMEM complete media (100 ml)

DMEM	87	ml
FBS	10	ml
1 M HEPES free acid	1	ml
100 Mm Sodium pyruvate	1	ml
100x Penicillin-streptomycin	1	ml

2) BMDMs media (100 ml)

L929 culture supernatant	20	ml
Complete DMEM media	70	ml
Horse serum	10	ml

3) Freezing media, A and B for BMDMs cryopreservation

3.1) Freezing media A (10 ml)

FBS	2	ml
DMSO	8	ml

3.2) Freezing media (10 ml)

FBS	2	ml
DMSO	2	ml
DMEM	6	ml

Preparation is to mix A: B at 1:1 ratio

4) Fetal bovine serum inactivation

Commercial fetal bovine serum were kept at -20°C and thawed at 4°C for overnight followed by inactivated at 56°C for 30 min in a water bath prior using

5) Immune complex preparation (10 µl for 1 ml of DMEM completed media)

10 µM Rabbit anti OVA (3-5 mg/ml) : 1xPBS : 1 µM OVA

1.7 µl : 3.3 µl : 5 µl

6) L929 culture supernatant

L929 cells were cultured in 8 ml of DMEM completed media in 5% CO_2, 37°C. The supernatant was collected when L929 cell was 70-80% confluent in a container. Supernatant was filtrated by using 0.2 µm filter and kept at 4°C for least than one month.

7) 1x PBS, pH 7.4 (1000 ml)

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	3.63	g
KH_2PO_4	0.24	g
DDW	1000	ml

Adjusted pH to 7.4 and autoclaved at 121°C

8) RIPA lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 7.4) (10ml)

1 M Tris-HCl pH 7.4	0.5	ml
0.5 M NaCl	3	ml
NP-40	0.5	ml
10% (w/v) sodium deoxycholate	0.5	ml
20% (w/v) SDS	0.05	ml
Sterile water	fill up to 10 ml	

9) 8% SDS-polyacrylamide gel (8ml)

DDW	4.236	ml
40% Acrylamide and Bis-acrylamide solution	1.6	ml
1.5 M Tris-HCl pH 8.8	2	ml
10% SDS	80	µl
10% APS	80	µl
TEMED	4	µl

10) 5% stacking gel (2ml)

DDW	1.204	ml
40% Acrylamide and Bis-acrylamide solution	0.25	ml
1 M Tris-HCl pH 8.8	0.504	ml

10% SDS	0.02	µl
10% APS	0.02	µl
TEMED	0.002	µl

11) PBS-T (1000 ml)

1xPBS	1000	ml
Tween20	0.5	ml

12) 1.5 M Tris-HCl, pH 8.8 (1,000 ml)

Trisma base	181.71	g
DDW up to	1000	ml

pH was adjusted into 8.8 with HCl

13) 1.5 M Tris, pH 6.8 (1,000 ml)

Trisma base	181.71	g
DDW up to	1000	ml

pH was adjusted into 6.8 with HCl

14) 5x Running buffer (1000 ml)

Trisma base	15.1	g
Glycine	94	g
SDS	5	g
DDW	1000	ml

15) Transfer buffer (1000 ml)

Trisma base	5.08	g
Glycine	2.9	g
SDS	0.37	g
DDW	1000	ml

16) 6x Gel loading buffer (10ml)

0.5 M Tris-HCl pH6.8	7	ml
SDS	1	g
Glycerol	3	ml
Bromophenol blue	0.001	g
ß-mercaptoethanol	0.5	ml

17) Blocking buffer for Western blot (3% skim milk) (100 ml)

PBS-Tween20	100	ml
Non-fat dry milk	3	g

18) ECL substrate of HRP

18.1) Coumaric solution

Coumaric acid	90	mM
DMSO up to	10	ml

The solution was aliquoted and stored at -20 °C

18.2) Luminol solution

Luminol	250	mM
DMSO up to	10	ml

The solution was aliquoted and stored at -20 °C

18.3) Solution A

100 mM Tris-HCl, pH 8.5	2.5	ml
coumaric acid solution	11	µl
luminol solution	23	µl

18.4)Solution B

100 mM Tris-HCl, pH 8.5	2.5	ml
30% H_2O_2	1.5	µl

19) Film developer and fixer

Each was diluted in tap water at 1:4 dilution

20) Coomassie blue stock solution (10 ml)

PhastGel™ Blue R	1 tablet	
Absolute methanol	120	ml
Distilled water	80	ml

One tablet of PhastGel™ Blue R was dissolved in 80 ml of distilled water and stirred for 5-10 mins. Next, 120 ml of methanol was added and the solution was

stirred until the dye was dissolved well. The solution was filtrated through Whatman filter paper No.1.

21) Coating buffer, pH 9.5 (1,000 ml)

NaHCO3	8.4	g
Na2CO3	3.56	g
DDW up to	1,000	ml

pH was adjusted to 9.5 and sterilized by 0.2 µm filter.

22) Blocking buffer for ELISA (10% FBS) (100 ml)

1xPBS	90	ml
FBS	10	ml

23) TMB buffer, pH 4.0 (1000 ml)

Tri-potassium citrate monohydrate	66.5	mg
Citric acid	39.38	g
DDW up to	1,000	ml

pH was adjusted to 4.0 by HCl

24) TMB substrate (for 10 ml TMB buffer)

TMB (3,3,5,5-tetramethylbensidine)	2.5	mg
DMSO	250	µl

Freshly prepared before used

25) TMB substrate solution

MB buffer	10	ml
TMB substrate	250	µl
30% H_2O_2	2.5	µl

26) 2N H_2SO_4 (stop solution) (500 ml)

Absolute H_2SO_4	27	ml
DDW	473	ml

27) FACS staining buffer (3% FBS) (100 ml)

1xPBS	97	ml
FBS	3	ml

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