The influence of Polycyclic aromatic hydrocarbons in FcgRIIb -/- lupus mice

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Microbiology
Medical Microbiology,Interdisciplinary Program
GRADUATE SCHOOL
Chulalongkorn University
Academic Year 2020

ผลกระทบของโพลีไซคลิกอะโรมาติกไฮโดรคาร์บอนในหนูลูปัส FcgRIIb -/-

น.ส.กัลยรัตน์ อุดมพรพิทักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ สหสาขาวิชาจุลชีววิทยาทางการแพทย์
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2563

Thesis Title	The influence of Polycyclic aromatic hydrocarbons in FcgRIIb -/- lupus mice
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Field of Study	Medical Microbiology
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กัลยรัตน์ อุดมพรพิทักษ์ : ผลกระทบของโพลีไซคลิกอะโรมาติกไฮโดรคาร์บอนในหนูลูปัส FcgRIIb -/- . (The influence of Polycyclic aromatic hydrocarbons in FcgRIIb -/- lupus mice) อ.ที่ปรึกษาหลัก : รศ. ดร.อัษฎาศ์ ลีฬหวนิชกุล

การกระตุ้นผ่านตัวรับ aryl hydrocarbon receptor (Ahr) ด้วยสารต่างๆรวมทั้ง Polycyclic aromatic hydrocarbons (PAHs) อันเป็นส่วนประกอบหลักของฝุ่นละอองขนาด 2.5 ไมครอน (PM2.5) อาจทำให้อาการของโรคลูปัสรุนแรงขึ้น 1,4-chrysenequinone (1,4-CQ) ซึ่งเป็นสารที่อยู่ในกลุ่ม PAHs จึงนำมาใช้ในการทดลองในโมเดลโรคลูปัสที่เกิดจากการขาด Fc gamma receptor IIb (FcgRIIb-/-)

การกระตุ้นเซลล์แมคโครฟาจด้วย 1,4-CQ เพียงอย่างเดียวไม่สามารถทำให้เกิดการอักเสบได้ แต่เมื่อกระตุ้นด้วย lipopolysaccharide (LPS) แล้วตามด้วย 1,4-CQ (LPS/1,4-CQ) ส่งผลให้มีการอักเสบที่รุนแรงกว่าการกระตุ้นด้วยสารเพียงอย่างใดอย่างหนึ่งโดยพิจารณาจากsupernatant cytokines (TNF-α, IL-6 และ IL-10) ยิ่งกว่านั้นเซลล์แมคโครฟาจที่ได้จากเซลล์หนู FcgRIIb-/- ซึ่งเป็นหนูที่เกิดโรคลูปัส มีการตอบสนองรุนแรงกว่าเซลล์จาก wild-type (WT) โดยพิจารณาจาก supernatant cytokines (TNF-α, IL-6 และ IL-10) และ การแสดงออกของปัจจัยกระตุ้นการอักเสบ (*NF-κB, aryl hydrocarbon receptor, iNOS และ IL-1β*) และ CD-86 ที่ผิวเซลล์ ยิ่งไปกว่านั้นการให้สาร 1,4-CQ เป็นเวลา 8 สัปดาห์โดยเริ่มให้ในหนู FcgRIIb-/- ที่มีอายุ 8 สัปดาห์เพิ่มความรุนแรงของโรคลูปัสโดยการทดสอบ anti-dsDNA, serum creatinine, proteinuria, endotoxemia และ gut-leakage (FITC-dextran)

ดังนั้นจึงสรุปได้ว่าการกระตุ้น PAHs ผ่านตัวรับ aryl hydrocarbon receptor (Ahr) อาจจะทำให้ความรุนแรงของโรคลูปัสในหนู FcgRIIb-/- แย่ลงและเกิดการตอบสนองต่อการอักเสบที่เพิ่มขึ้น ซึ่งเป็นไปได้ว่าเกิดจากการขาดตัวยับยั้งชนิด FcgRIIb ซึ่งเป็นตัวยับยั้งเพียงตัวเดียวในกลุ่ม FcgR ในเซลของหนูลูปัสเหล่านี้ ข้อมูลเหล่านี้ชี้ให้เห็นว่าผู้ป่วยโรคลูปัสอาจมีความเสี่ยงต่อภาวะแทรกซ้อนของมลพิษทางอากาศมากกว่าในคนปกติ

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....
ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6187110520 : MAJOR MEDICAL MICROBIOLOGY

KEYWORD: FcgRIIb deficient mice, systemic lupus erythematosus, aryl hydrocarbon receptor, air pollution, Polycyclic aromatic hydrocarbons

Kanyarat Udompornpitak : The influence of Polycyclic aromatic hydrocarbons in FcgRIIb -/- lupus mice. Advisor: Assoc. Prof. ASADA LEELAHAVANICHKUL, M.D., Ph.D

The activation of aryl hydrocarbon receptor (Ahr) through polycyclic aromatic hydrocarbons (PAHs), the major components of particulate matter at 2.5 micron (PM2.5) in the polluted air, might aggravate inflammation and lupus activity. Hence, 1,4-chrysenequinone (1,4-CQ), a substance in the PAHs group, was tested in a lupus model from FcgRIIb deficiency (FcgRIIb-/-) using macrophages and mice.

Although the activation by 1,4-CQ alone was unable to induce inflammation, the pre-conditioning by lipopolysaccharide (LPS), a representative inflammatory-activator, before 1,4-CQ (LPS/1,4-CQ) induced more predominant inflammation in macrophages when compared with LPS or 1,4-CQ activation alone as determined by supernatant cytokines (TNF-α, IL-6 and IL-10). Additionally, the activation in FcgRIIb-/- macrophages induce the more prominent inflammation than the wild-type (WT) cells, as determined by supernatant cytokines (TNF-α, IL-6 and IL-10), expression of inflammatory-genes (*NF-κB, aryl hydrocarbon receptor, iNOS, IL-1β*) and cell-surface CD-86, possibly due to the lack of inhibitory-FcgRIIb. Moreover, 8-wk-administration of 1,4-CQ started in 8-week-old FcgRIIb-/- mice, a genetic-prone lupus model, enhanced lupus severity as indicated by anti-dsDNA, serum creatinine, proteinuria, endotoxemia, and gut-leakage (FITC-dextran).

In conclusion, the Ahr activation by PAHs worsened lupus severity in FcgRIIb-/- mice possibly through the increased inflammatory responses due to the loss of the inhibitory-FcgRIIb. These data suggest that patients with lupus are possibly more vulnerable to the air pollution than the healthy persons.

Field of Study:	Medical Microbiology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

ACKNOWLEDGEMENTS

The author would like to thanks my adviser Asada Leelahavanichkul, MD, PhD, Medical Microbiology, Interdisciplinary Program, Graduate School, Chulalongkorn University and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

Kanyarat Udompornpitak

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CHAPTER I

INTRODUCTION

Particulate matter (PM) is an important component of air pollution the possible adverse effect upon the healthiness of people in the area (1, 2). According to the World Health Organization (WHO), air pollution causes approximately 82% of the premature deaths (3). More than 80% of people living in urban areas that monitor air pollution have experienced air quality levels that exceed the WHO limits (4). Indeed, Thailand has experienced the increasing air pollution problem that was possibly associated with the negative health impacts (5). PM is a general indicator for air pollution (6). It affects more people than any other pollutions (7). Because i) the impact of most particulate-bound polycyclic aromatic hydrocarbons (PAHs) and nitro-polycyclic aromatic hydrocarbons (NPAHs) contained in PM2.5 against people healthiness is mentioned (8) ii) the particles with a diameter less than 10 and 2.5 microns (PM10 and PM2.5) can penetrate deeper into the lungs and could be absorbed directly into blood circulation, respectively, thus chronic exposure to PM might enhance the risk of respiratory abnormalities, including lung cancer and cardiovascular diseases, respectively (3, 9-12). The major components of PM are PAHs, NPAHs, sulfate, nitrates, ammonia, sodium chloride, black carbon, and mineral dust (2). Among them, the great concern in one of these major components of PM2.5 is PAHs, because of the toxicity of this material as human carcinogenicity and/ or

inflammatory activator (5, 13). The total summation of the main combustion-derived PAHs concentrations (COMPAHs) is more than 50% of the total components in $PM_{2.5}$ (14). On the other hand, aryl hydrocarbon receptor, the receptor encoded by *Ahr* gene, is responsible to induce biological response to environmental pollutants. As such, PAHs or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were frequently used as a representative of PM2.5 (15). Accordingly, PM2.5 might be associated with the adverse effects in patients with underlying diseases, while PM2.5 might have a lesser influence upon the normal host. As such, the impact of PM2.5 in patients with systemic lupus erythematosus (SLE), a common autoimmune disease, might be more prominent than other people with the normal health. Despite several causes of SLE, the functional defect of FcgRIIb, the only inhibitory receptor among FcgR family, is mentioned as one of the genetic defects in Asian population that leads to SLE (16-18). Indeed, the prevalence of the dysfunction-polymorphism of *FcgRIIb* gene in Thailand is common (19). Also, SLE, referred to as "lupus", is an autoimmune disease in which the body's immune system mistakenly attacks healthy tissue in many parts of the body. Symptoms of SLE vary between people and may be mild to severe. In addition, SLE patients have an increased risk of being hospitalized for pneumonia when compared with the general population and *Pseudomonas aeruginosa* infection is common in lupus patients. Additionally, spontaneous endotoxemia has also been reported in lupus patient, despite the data about the effects of gastrointestinal (GI) leakage in lupus are still limited. Interestingly, elevated

blood endotoxin, a component of the gram-negative bacterial cell wall, seems to be common in patients with SLE. As the GI tract is the endogenous source of endotoxin, endotoxemia in active lupus may be due to gut leakage (20, 21).

On the other hand, the cause of SLE is not clear. It is thought to involve genetic abnormalities together with the environmental factors. In the previous studies, PM2.5 is associated with activity of systemic autoimmune rheumatic diseases (SARD), including SLE, in an urban Canadian cohort (22). The odd ratio of SARD were elevated among those with higher exposures to air pollution (particulate matter) in two other Canadian provinces as well (23, 24). It has been documented that several major components of air pollution, including trace elements and polycyclic aromatic hydrocarbons, are also associated with the prevalence of SLE. However, the impact of air pollution on the SLE disease activity is still unclear (25). Interestingly, FcgRIIb-/- mice, one of the representative lupus models, demonstrated full-blown active lupus at 40-week-old (21). Hence, our study aims to investigate the impact of PAHs, as Ahr activator upon macrophages and mice from WT and FcgRIIb-/- group. While advanced understanding of molecular mechanisms of air pollution affecting immune responses may pave the way for the better prevention and treatment for autoimmune diseases, this study may contribute a novel insight on the biological role of air pollutant in SLE patients.

CHAPTER II

HYPOTHESIS AND OBJECTIVE

Hypothesis

Polycyclic aromatic hydrocarbons increase the inflammatory response in FcgRIIb -/- macrophage upon activation of the Aryl hydrocarbon receptor.

Objective

1. To *in vitro* investigate the influence of PAHs, as Ahr activator, upon FcgRIIb -/- lupus macrophages pre-activated with or without LPS.

2. To *in vivo* investigate the influence of PAHs upon FcgRIIb -/- lupus mice primed with or without LPS.

Research question

1. Do PAHs with or without LPS pre-activation regulate the inflammatory response of FcgRIIb -/- macrophages *in vitro*?

2. Do PAHs with or without LPS pre-activation regulate the inflammatory response of FcgRIIb -/- mice *in vivo*?

CHAPTER III

LITERATURE REVIEW

Air pollution

Particulate matter (PM) is one of the primary pollutants of the environment which is a worldwide problem (26). Epidemiological studies have confirmed the association between the exposure to PM and increased rate of morbidity and mortality (27, 28). However, data of mechanism underlying PM-related health effects are still limited (29). Indeed, it is obvious that air pollution contributes a detrimental factor to our health. With the rapid development of industry, transport, energy, power generation, and agriculture, the environmental pollution is becoming a stronger threat to the world. In 2016, air quality in more than 91% area around the world does not meet the criteria of pollution flowing the World Health Organization (WHO) for a healthy environment standard (30), which has drawn the worldwide attention. Air pollution is described as a complicated mixture, comprising particulate matter (PM), carbon monoxide, lead, nitrogen dioxide, ozone, sulfur dioxide and so on. More importantly, PM2.5, the small PM with diameter less than 2.5 μm, is now regarded as one of the most harmful factors to our health. According to the data analysis of WHO, in 2016 there were approximately 4.2 million people suffered from air pollution, leading to shorter lifespan, which is mostly resulted from the PM2.5 (3). Many studies illustrated that air pollution and PM2.5 can penetrate deeper into the

lungs and could be absorbed directly into blood circulation, therefore, chronic exposure to PM might enhance the risk of respiratory abnormalities, including lung cancer and cardiovascular diseases **(Fig 1)** (3, 9-12, 30, 31).

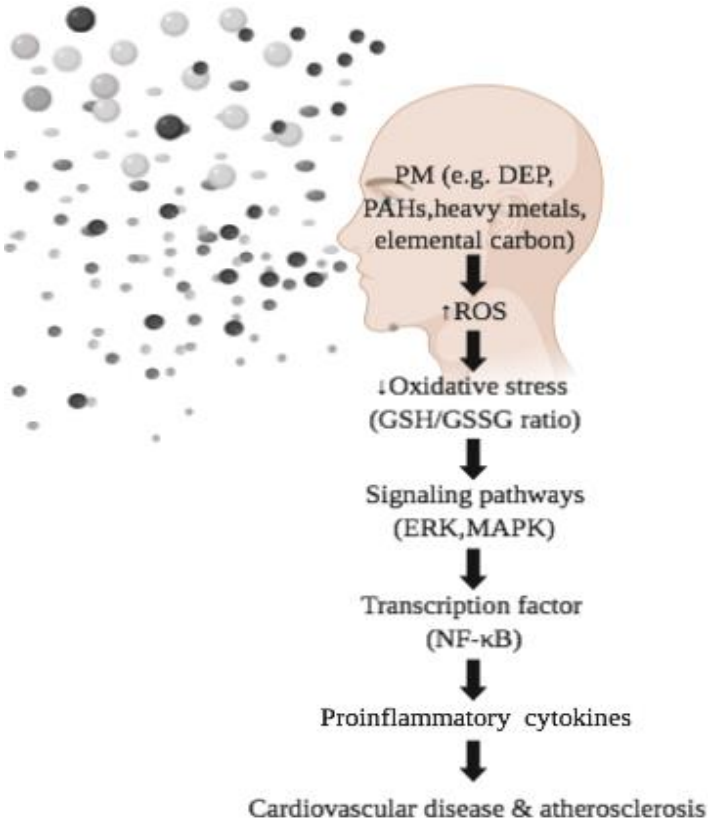


Figure 1. PM induced cardiovascular and Atherosclerotic effects.

(Image, using BioRender.com, is modified from reference number 10; Lawal, 2017 (10).)

PM2.5

Particle matter (PM) is described as any particle suspended in the air. According to the size of the particle, it is classified into PM10, PM2.5, and PM1.0 (32-35). Fine particulate matter (PM2.5) has become a major public health concern because of their adverse effects on health. Lungs are considered the primary organ affected as PM2.5 can penetrate deep into the respiratory tract and reach alveolar ducts (36, 37). Fine particulate matter (PM2.5) has been closely associated with increased rate of morbidity and mortality, especially in lung cancer, worldwide (38, 39). Several potential mechanisms have been involved in the adverse effects of PM2.5, including cytotoxicity induced by oxidative stress (40), oxidative DNA damage, mutagenicity, micronucleus formation and pro-inflammation stimulation (41, 42).

Penetration ability of particle pollution with different size:

- PM10: Particles larger than 10 μm generally was caught in the nose and throat, never entering the lungs. Particles smaller than 10 μm can get into the large upper branches just below the throat where they are caught and removed by coughing and spitting or by swallowing.
- PM5: with the size smaller than 5 μm, the particles can get into the bronchial track at the top of the lungs.

- PM2.5: with the size smaller than 2.5 μm, particles can enter alveolar of the lung. These particles soluble in water will pass directly into alveolar capillaries and dissolved in blood whereas insoluble particles are stuck in the deep lung (1).

Sources of PM

Particulate pollution can be generated directly or indirectly from a numerous source such as vehicles, agriculture, industry, deforestation, chemical waste (2). These particles are of variation in sizes and shapes and can be made up of hundreds of different chemicals. Most particles are generated in the atmosphere as a consequence of sophisticated reactions of chemicals such as nitrogen oxides and sulfur dioxide (43).

The previous studies illustrated biological effect of PM on human by the contaminants adsorbed on the particles being causal reason for multiple health issues (44, 45). Moreover, several researches indicated that the PM composition may immensely alters upon seasons, sources and regions (46). PM2.5 samples in urban areas is reported with chemical components, including inorganic ions, total carbon, elements from anthropogenic sources, polycyclic aromatic hydrocarbons (PAHs), and biological products (47). Among all, PAHs - one of these major components of PM2.5 is the most harmful component due to its toxicity on carcinogenicity and/ or

inflammation (5, 7, 25). PAHs present in air because of the release from commonly sources, such as transportation emissions and industry process, and cigarette smoke (25).

Thus, PAHs are spread out largely and persistently in the atmosphere (48). Many toxicities, such as atherogenesis or carcinogenicity, results from PAHs (49, 50). Meanwhile, PAHs are well evidenced to induce the toxic impacts by provoking Ahr (51-53). Nevertheless, specific mechanisms uncovering for the toxicities are not yet elucidated. Ahr is identified as receptor that controls gene expression, including CYP1A1, CYP1A2, and CYP1B1 (54). Accordingly, CYP1A1 is an enzyme serving as carcinogenic activators such as B[*a*]P (55). The induction of CYP1A1 includes B[*a*]P and MC through Ahr activation after exposure to PAHs (54, 56). Several reports claimed that PAHs can activate metabolism by CYPs, encompassing CYP1A1, which is a necessary step for vascular atherosclerosis (16, 49, 50).

Aryl hydrocarbon receptor (Ahr)

Fundamental Information of the Ahr

The Ahr is cytosolic receptor in basic helix–loop–helix/Per-ARNT-Sim (bHLH-PAS) transcription factor families (57). It is reported that TCDD, benzo(a)pyrene, and polycyclic aromatic hydrocarbons (PAHs) impact on cell by directly binding to Ahr (56). Also, Ahr is activated by other cytosolic ligands from exogenous or endogenous factors (58, 59).

The basic structure of the Ahr protein consist of three parts: the bHLH motif, a Q-rich domain, and PAS domains. The fundamental domain of the bHLH motif is at the N-terminal region for promote target gene expression while the PAS domains play a role in a heterozygous protein complex by integrating the Ahr nuclear translocator (ARNT) and the ligand. Q-rich domain is at the C-terminal region of the protein, which help for the recruitment and activation of transcription of the motif **(Fig 2).**

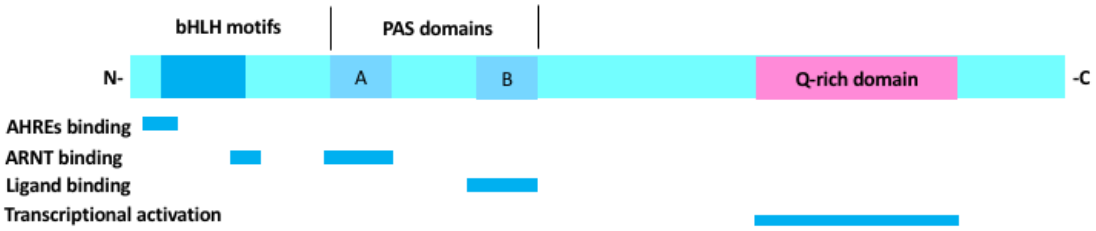


Figure 2. Functional structure of the aryl hydrocarbon receptor (Ahr).

(Image, using BioRender.com, is modified from reference number 60; Xue, Fu, 2018 (60).)

Without the present of ligands, Ahr is suspended in cellular cytoplasm (61-63). Upon binding to ligands such as PAHs, kynurenine, TCDD, 6-formylindolo[3,2-b]carbazole (FICZ), and 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), the Ahr complex is initiated with activation. This action takes place by translocation of the complex into nucleus, separated from chaperone proteins, and interacting with ARNT. The chaperone proteins play a protective role for the Ahr from proteolysis and maintain appropriate construction for the binding site (64). The Ahr–ARNT heterodimer is associated with other signaling mediators (such as transcriptional factors, histone acetyltransferases, and chromatin remodeling factors) and ultimately activates DREs or AHREs for promote transcriptional regulation (65, 66). Classical target genes of Ahr encompass Ahr repressor, cytochrome P450 (CYP)1A1, CYP1A2, and CYP1B1.

Moreover, Ahr presents in most of tissues in humans and expressed vastly in the lungs, liver, and brain (67, 68). In the immune cells, Ahr presents in macrophages, dendritic cells, T cells, B cells, natural killer (NK) cells, epithelial cells, Langerhans cells, microglia **(Fig 3)** (18, 19, 69-73).

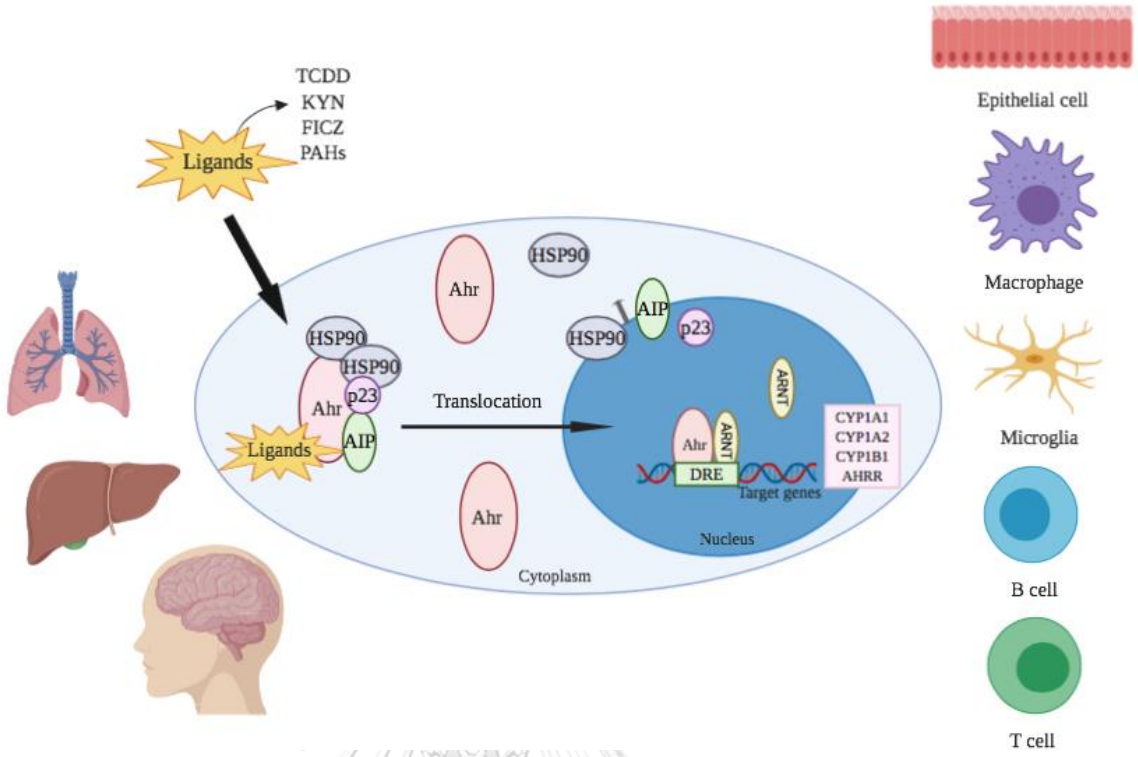


Figure 3. Mechanism of activation of the aryl hydrocarbon receptor (Ahr).

(Image, using BioRender.com, is modified from reference number 60; Xue, Fu, 2018 (60).)

Ahr Ligands

The Ahr is inhibited or activated by many kinds of exogenous and endogenous ligands. Differences in ligand types interacting with the Ahr is capble of inducing different effects **(Fig 4)** (74).

Exogenous/Xenobiotic Ligands

The well-documented exogenous/xenobiotic ligands with the high affinity to Ahr are environmental pollutants such as PAHs, polychlorinated biphenyls, and

halogenated aromatic hydrocarbons. A typical exogenous ligand for the Ahr is PAHs, an environmental pollutant with high toxicity. PAHs is a prominent epigenetic carcinogen and a potent tumor inducer (75, 76). Activation by PAHs can cause various toxicity (hepatotoxicity, immunotoxicity, cardiotoxicity, tumor promotion reproductive toxicity, teratogenesis, dermal toxicity wasting syndrome, and endocrine disruption and lethality) (77). Meanwhile, mice without Ahr (AhR−/−) are not sensitive to the toxic influence of PAHs or PAHs-like substances (78-80).

Endogenous Ligands

The endogenous stimuli of Ahr (81) in mammalian cells, such as indirubin, indigo from products of human urine (82), the respiratory secretion (81), and the metabolites from arachidonic acid, and equilenin (83).

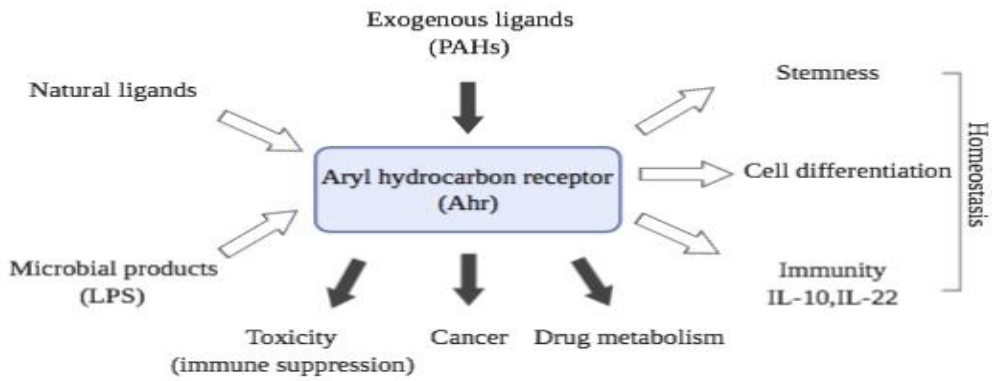


Figure 4. The Ahr functions as a pivotal sensor connecting external and internal environments. (Image, using BioRender.com, is modified from reference number 84; Kawajiri and Fujii-Kuriyama, 2017 (84).)

Most of the natural stimuli which is Ahr agonist depend on dioxin-response elements (DRE) Indirubin, indigo compete with PAHs for occupying receptor and promote activity of CYP1A1 monooxygenase (85, 86). Equilenin is an estrogen metabolite that activate Ahr in HepG2 cells (liver cells) as demonstrated by an increase in expression of CYP1A1 mRNA and DRE-induced reporter signals (87). Endogenous Ahr ligands from the derivations of arachidonic acid consist of prostaglandins (PGs) and lipoxin A4. Lipoxin A4 activates Ahr by inducing expression of CYP1A1 and CYP1A2 monooxygenases as investigated in mouse hepatic cells (88). Furthermore, effect of PG-G2 was determined hepatocytes by dose–dependent characteristics, suggesting that it is possible to augment DRE-mediated transcription although PG-G2 (89). In addition, the metabolites from heme are considered as endogenous Ahr ligands whose bilirubin was the most crucial activator. It is reported that enzymatic function of CYP1A1 might be regulated directly by bilirubin through Ahr-involved cascades in rodent hepatocytes (90). Amongst ligand mentioned above, emerging evidence shows that 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) and kynurenine are novel potential candidate on investigation currently.

Immune system

The immune system fights germs and foreign substances on the skin, in the tissues of the body and in bodily fluids such as blood. The immune system is made up of two parts: the innate, (general) immune system and the adaptive (specialized) immune system **(Fig 5).** These two systems work closely together and take on different tasks (91).

The innate immune system: Fast and general effectiveness

The innate immune system is the body's first line of defense against germs entering the body. It responds in the same way to all germs and foreign substances, which is why it is sometimes referred to as the "nonspecific" immune system. It acts very quickly. The innate immune system consists of physical barriers (skin, epithelium, saliva, etc.) as well as immunological barriers in the form of various immune cells (monocytes, macrophages, neutrophils, etc.) that recognize molecular patterns or motifs in pathogens through “pattern recognition receptors” such as Toll-like receptors, NOD-like receptor proteins, C-type lectin receptors and RIG-1like receptors. The ligation of these receptors triggers a proinflammatory signaling that orchestrates the early response to infection and leads to subsequent activation of cells of the adaptive immune system (T and B lymphocytes).

The adaptive immune system*:* Specific responses to the germs

The adaptive immune system takes over if the innate immune system is not able to destroy the germs. It specifically targets the germ that is causing the infection. But to do that it first needs to identify the germ. This means that it is slower to respond than the innate immune system, but when it does it is more accurate. It also has the advantage of being able to "remember" germs, so the next time a known germ is encountered, the adaptive immune system can respond faster. This memory is also the reason why there are some illnesses you can only get once in your life, because afterwards your body becomes “immune.” It may take a few days for the adaptive immune system to respond the first time it comes into contact with the germ, but the next time the body can react immediately. The second infection is then usually not even noticed or is at least milder. The adaptive immune system is made up of: T lymphocytes in the tissue between the body's cells, B lymphocytes, also found in the tissue between the body's cells, and Antibodies in the blood and other bodily fluids (92, 93).

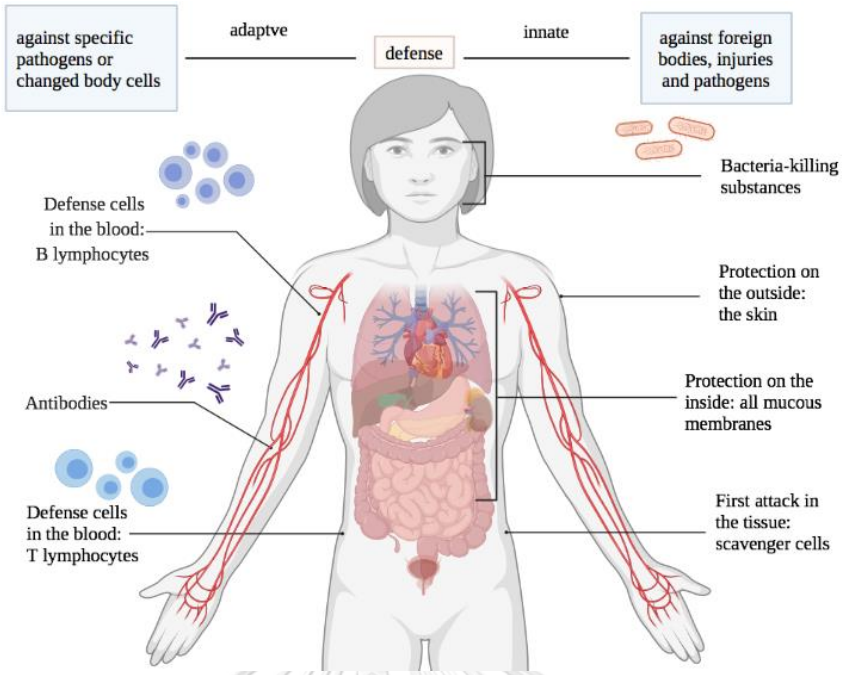


Figure 5. The innate and adaptive immune systems.

(Image, using BioRender.com, is modified from reference number 91; InformedHealth, 2020 (91).)

Macrophages

Macrophages are a diverse group of white blood cells known for eliminating pathogens through phagocytosis. In the past, macrophages are classified by the organ in which they are found: Kupfer cells in the liver, Langerhans cells in the skin, microglia in the brain and spinal cord, osteoclasts in the bone. Macrophage polarization is a process by which macrophages adopt different functional programs in response to the signals from their microenvironment (94). This ability is connected to their multiple roles in the organism control. Macrophages are powerful effector

cells of the innate immune system, but also important in removal of cellular debris, embryonic development, and tissue repair.

According to the activating state and functions of macrophages, they can be divided into M1-type (classically activated macrophage) and M2-type (alternatively activated macrophage) **(Fig 6).** The role of M1 macrophages is to secrete pro-inflammatory cytokines and chemokines, present antigens, and thus participate in the positive immune response and function as an immune monitoring. The main pro-inflammatory cytokines from macrophages are IL-6, IL-12, and TNF-α. M2 macrophages mainly secrete arginase 1 (Arg1), IL-10 and TGF-β and other anti-inflammatory cytokines, which have the function of reducing inflammation and contributing to the enhanced tumor growth and Immunosuppressive function. It plays an important role in wound healing and tissue repair.

Macrophages involve in the elimination of pathogens in tissues. When activated, macrophages can engulf and kill pathogenic microorganisms, release pro-inflammatory factors, collect, and activate lymphocytes to induce an adaptive immune response.

Macrophage Markers

CD68 and CD11b are total markers of macrophages. For M1 and M2 macrophages, they have specific markers.

- M1 Macrophage Marker

M1 can choose CD80, CD86, CD64, CD16 and CD32 as markers. In addition, the expression of nitric oxide synthase (iNOS) in M1 can also serve as phenotypic markers.

- M2 Macrophage Marker

CD163 and CD206 are major markers for the identification of M2 macrophages. Related surface markers for M2-type cells also contain CD68. Compared with marker CD68, CD163 is more selective to macrophages, so CD163 can be used as a highly specific marker for M2-type macrophages. In addition, arginase 1 (Arg1) and DECTIN-1 are also ideal phenotypic indicators for the identification of M2 macrophages. Studies have also shown that FIZZ1, Ym1 and Ly6C can also be used as surface markers associated with M1 or M2 macrophage subpopulations.

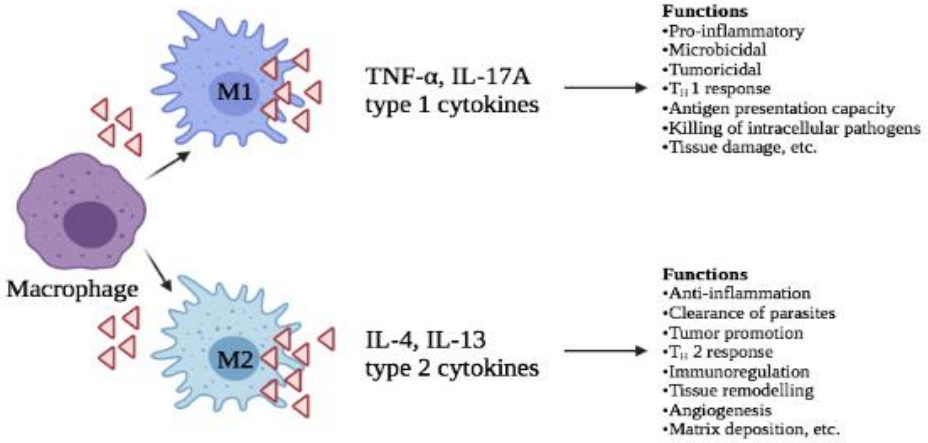


Figure 6. Macrophage polarization and specific function of M1 and M2 macrophage.

(Image, using BioRender.com, is modified from reference number 94; CUSABIO, 2020 (94).)

The Aryl hydrocarbon receptor and Macrophage

Inflammation is one of the innate immune responses. To limit the undesirable consequences of excessive inflammation, factors that modulate the initiation phase and the resolution phase of inflammation can determine the nature of the inflammatory response (95). Ahr is a ligand-activated transcription factor that was initially recognized as a receptor mediating the pathologic effects of dioxins and other pollutants (96). Recent studies have identified the molecular functions of Ahr in the immune system during a steady state and during infection and inflammatory state (97-100). Ahr involves in LPS-induced inflammatory gene expression (101). Ahr deficient (Ahr-KO) mice are hypersensitive to LPS-induced septic shock, mainly because of macrophage dysfunction. Consistent with the enhanced susceptibility to LPS treatment, Ahr-KO mice markedly increase plasma levels of IL-1β, IL-18, IL-6, and TNF-α (102). Activated Ahr also plays a central role in limiting endotoxin-triggered inflammation, resulting in the establishment of endotoxin tolerance (103). Emerging studies reveal that Ahr has a pivotal role to play in modulating immune responses and that activation with Ahr-involved toxicants leads to pathogenesis of immune disorders (104, 105).

Inflammatory responses mediated by macrophages are a part of the innate immune system (106). Macrophages are important effector cells of innate immunity, with a pivotal role in host defense against intracellular pathogens (107). The roles of

Ahr in the differentiation and other functions of specific T-cell subpopulations and B cells in adaptive immune response are well known (98). However, although numerous studies have addressed the modulatory effect of Ahr in innate immune cells, such as dendritic cells (DCs), neutrophils and natural killer cells (108), the exact role of Ahr in macrophage function remains to be elucidated. Increasing evidence have also demonstrated roles for Ahr in the regulation of inflammation and inflammatory cytokines. Ahr negatively regulated IL-6 production in macrophages following LPS stimulation (18, 19). Ahr activation also inhibits caspase-1 activation and subsequent IL-1β secretion in macrophages (109). IL-10 is an immunoregulatory cytokine with a crucial role in ameliorating immunopathology and preventing inflammatory responses, which leads them to hypothesize that Ahr may also regulate IL-10 expression in inflammatory macrophages in innate immunity (110).

Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a kind of autoimmune disease with a strong genetic predisposition caused by complicated factors, it is also considered as an inflammatory disease caused by the mediation and deposition of immune complexes (ICs), leading to damage of multiple organs (111, 112).

In SLE, the excessive immune system recognizes self-antigens and attacks their own cells, resulting in tissue damages and organ failure. It can impact on multiple organs and system such as skin, joint, brain, kidney, lung to name but a few **(Fig 5)**. The etiology and pathogenesis of SLE are multiple and unclear, but which are associated with various factors like environmental, hormonal, genetic elements (113). SLE affects women more than men. Women also may experience more severe symptoms during pregnancy and with their menstrual periods. No cure for SLE exists. The goal of treatment is to ease symptoms. Treatment can vary depending on how severe your symptoms are and which parts of your body SLE affects.

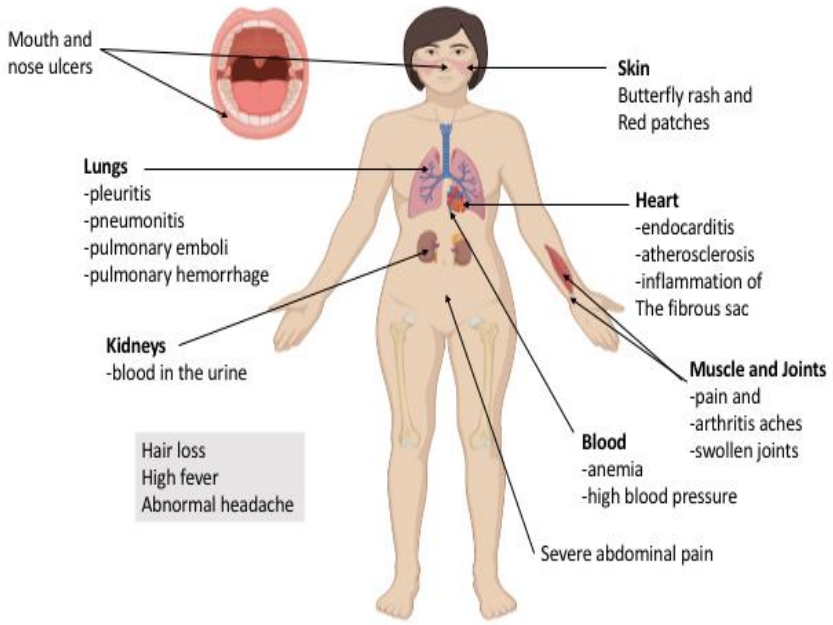


Figure 7. Systemic lupus erythematosus (SLE).

(Image, using BioRender.com, is modified from reference number 114; MCVitamins, 2019 (114).)

FcgRIIb

FcgRIIb or CD32B is well-known as the only inhibitory receptor in the IgG FcR family (115, 116). FcgRIIb is involved with an ITIM in domain at cytoplasm along with their counterpart as tyrosine-based activation signals for maintaining balances (117). For instance, after stimulation with BCR, BCR binds with FcgRIIb through IgG immune complexes, FcgRIIb activation leads to SHIP activation and downregulates B cell proliferation. Therefore, FcgRIIb exerts crucial feedback mechanism to compensate IgG production (118). With macrophages, it is evident that FcgRIIb acts as inhibitor of FcgRIIa (CD32A) and FcgRIIIa (CD16A) that compromises the phagocytosis that activated by FcgRIIa and FcgRIIIa (116, 119, 120). Moreover, the immunomodulatory

effect of FcgRIIb is observed in mast cell as it has a considerable affinity to FC epsilon receptor of IgE (121, 122).

In line with this, inhibitory role of FcgRIIb also showed in dendritic cells as the depletion of FcgRIIb signaling is likely to boost maturation and activation of myeloid DC (mDC) (123-125). However, FcgRIIb on dendritic cell accelerates function of antigen presenting then augmenting T cell activation, leading to the more humoral responses (126, 127). Hence, the versatile effect of FcgRIIb is dependent on different cell types.

In parallel, FcgRIIb-associated pathway play an important role in mediating two opposite immune phenomena, including autoimmune or tolerance response. It is proved that FcgRIIb-knockout mouse show a high level of Ig and a significantly high anaphylactic reaction (128). Indeed, plenty of immune response in FcgRIIb-knockout mouse was well-observed with lupus symptoms, including glomerulonephritis or autoantibodies deposition in several organs (129). Additionally, B-cells are engineered with over-expression of 40% FcgRIIb that is capable of manifesting tolerance maintenance and autoimmunity impediment (130). Taken together, FcgRIIb is a mechanism for regulating lupus-involved autoimmunity.

On the other hand, FcgRIIb is also found in human Chr.1q23 as lupus-susceptibility locus (131). Nevertheless, by virtue of the limitation of antibody to distinguish human FcgRIIb and human FcgRIIa (which is exclusive in human but not found in mouse), effort to optimize antibody recognizing FcgRIIb but not FcgRIIa in

human has been made. Moreover, this study explored that there is lower level of FcgRIIb expressed on memory and plasma B cell in SLE patient in comparison with heathy clusters. Considered, reduction of FcgRIIb in context of SLP disease or via genetic engineering could result in considerably excessive activation of B cell, suggesting a prominent inhibitory role of FcgRIIb for compromise autoimmunity in SLE patients (132).

Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria **(Fig 8).** Lipopolysaccharide is localized in the outer layer of the membrane and is, in non-capsulated strains, exposed on the cell surface. Lipopolysaccharides are heat stable endotoxins and have long been recognized as a key factor in septic shock (septicemia) in humans and, more generally, in inducing a strong immune response in normal mammalian cells. Model for bacterial infection in hosts with several common organisms such as *Streptococcus pneumoniae*, *Pseudomonas aeruginosa (hospital acquired pneumonia), Haemophilus influenzae (community acquired pneumonia)* (133) and Intriguingly, patients diagnosed of SLE was vulnerable to pneumonia and *Pseudomonas aeruginosa* infection spontaneous endotoxemia was well observed in patients. Moreover, increasing blood endotoxin LPS is reported to be common in patients with SLE. As the GI tract is the endogenous source of endotoxin, endotoxemia in active lupus may be due to gut leakage (20, 21). Hence, the activation of LPS in FcgRIIb-/- mice and cells could be used for the representative model of gram-negative bacterial infection in lupus.

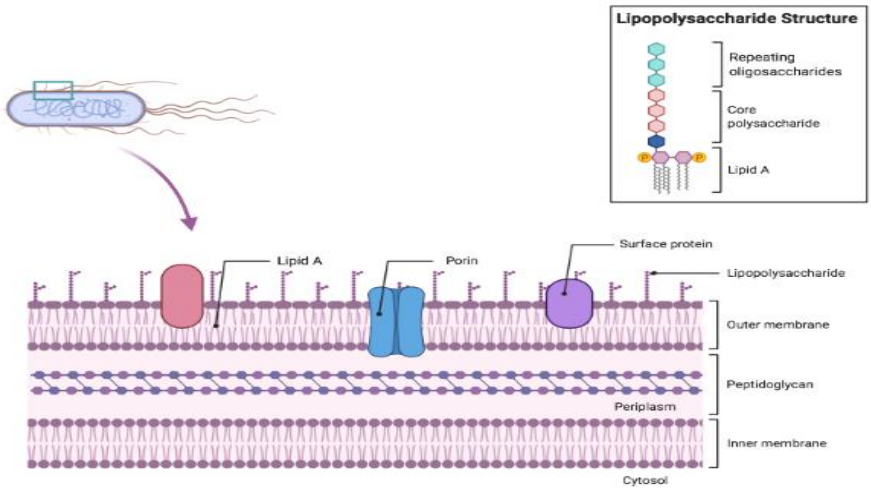


Figure 8. Cell Wall of Gram-negative bacteria. (Image, using BioRender.com)

Impact of PM2.5 on pathogenesis of systemic lupus erythematosus

SLE is characterized by a chronic autoimmune response while the prevalence is reported with ratio of 1 in 2,500 women worldwide (134, 135). Noticeably, any organs can be damaged that might induce the life-threatening manifestations (136, 137). Numerous possible exogenous triggers of lupus by the pollutions are mentioned with the limited data (24, 135, 138, 139) and the impact of air pollutants on SLE pathogenesis has never been studied. Particulate matters (PM) in air pollution contributes to impact on the immunity like those of breathing cigarette silica and smoke, leading to a wide range of chronic diseases such as chronic bronchitis, asthma, cardiovascular disease, laryngitis, and lung cancers (23, 140-146). Nevertheless, particulate matter is associated with the exaggeration of systemic autoimmune rheumatic diseases (SARD) (138, 147) with the controversial arguments

on its role of SLE pathogenesis partly due to the socio-demographic factors in terms of particulate air pollution (148). The previous studies, PM2.5 associated with activity of systemic autoimmune rheumatic diseases (SARD), including systemic lupus in an urban Canadian cohort (22). The odds of SARD are elevated among those with higher exposures to air pollution particulate matter in two other Canadian provinces as well (23, 24). It has been documented that several major components of air pollution, including trace elements and polycyclic aromatic hydrocarbons, are also associated with the prevalence of systemic lupus erythematosus (SLE). However, the impact of air pollution on the SLE disease activity is still unclear (31). Immune cells, including macrophages which can be affected by PM, are able to produce inflammatory signals upon LPS stimulation, including interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and IL-1β (149). PM also stimulates migration of monocytes into the tissue (150).

Moreover, lymphoblasts or immune cell precursors exposed to diesel-emitted particles revealed a sharp response by increasing reactive oxygen species, IL-8, and IL-6 (151). This indicates mechanisms in which PM could induce autoimmune diseases like SARDs, via exaggerate immune responses (22, 23). Ahr activated upon different exogenous and endogenous stimuli lead to the divergent impacts **(Fig 4);** consist of carcinogenesis, drug metabolism, cell development, cell homeostasis, host defenses, cell differentiation, stem cell maintenance, and immune response (84). Because Ahr is the dominated receptor accounting for the PAHs activation, a main component of PM2.5, thus the Ahr-activator is frequently used for studying the

impact of PM2.5 (152-158). Although Ahr activation upon wild-type macrophages is thoroughly studied, the study upon the abnormal hosts might be different. Indeed, PM2.5 might be related to detrimental effects in lupus patients with some explaining diseases, while the lesser influence of PM2.5 upon the normal host. As such, systemic lupus erythematosus (SLE), a common autoimmune disease, due to the functional defect of FcgRIIb, the only inhibitory receptor among FcgR family, is mentioned (129, 159, 160) and the prevalence of the dysfunction-polymorphism of FcgRIIb gene in Thailand is common (161). Interestingly, FcgRIIb-/- mice, a representative lupus model, demonstrated full-blown active lupus at 40-week-old (21). Hence, we studied the impact of PAHs activator upon macrophages and mice from WT and FcgRIIb-/- group.

CHAPTER IV

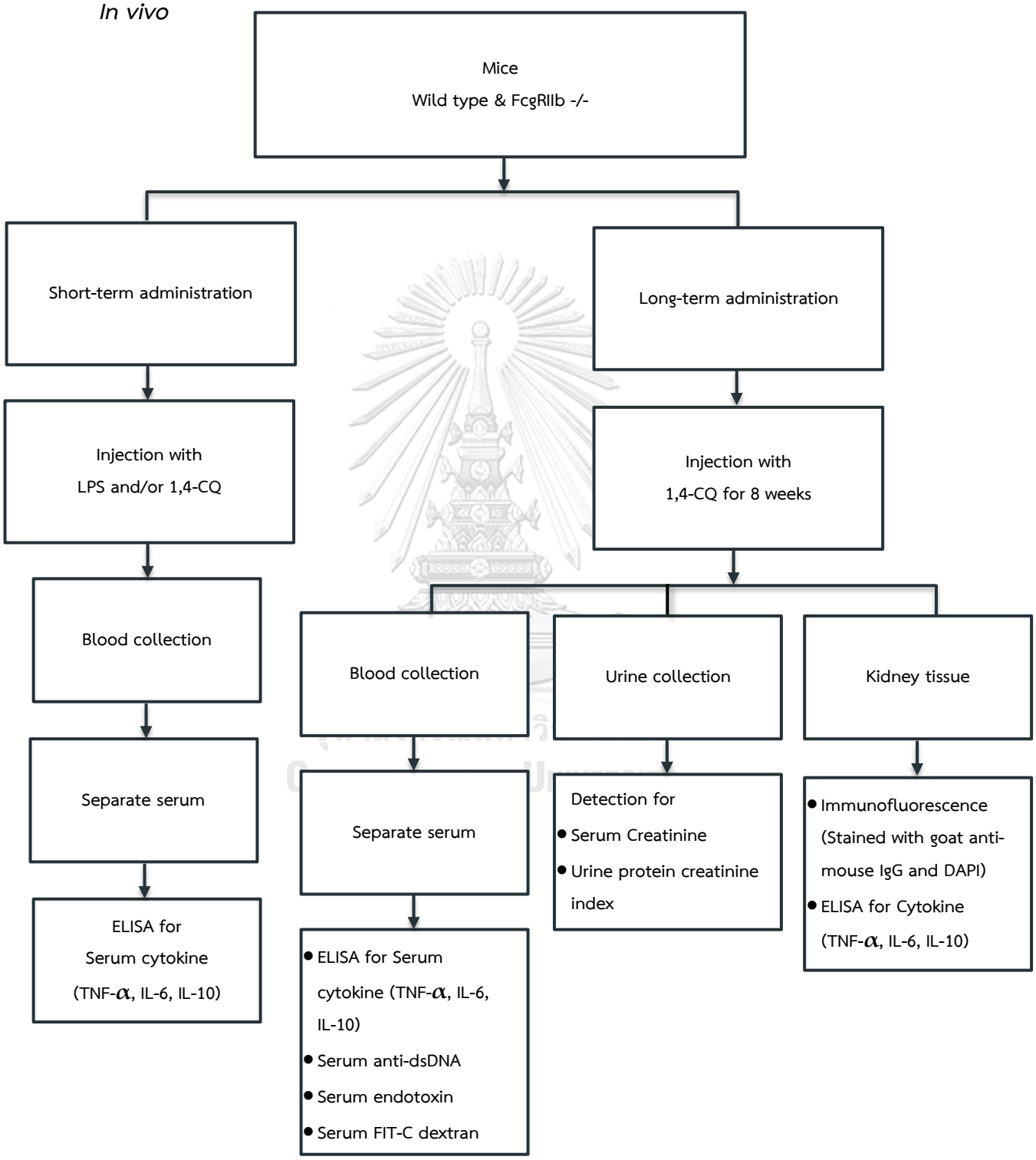
METHODOLOGY

Conceptual framework

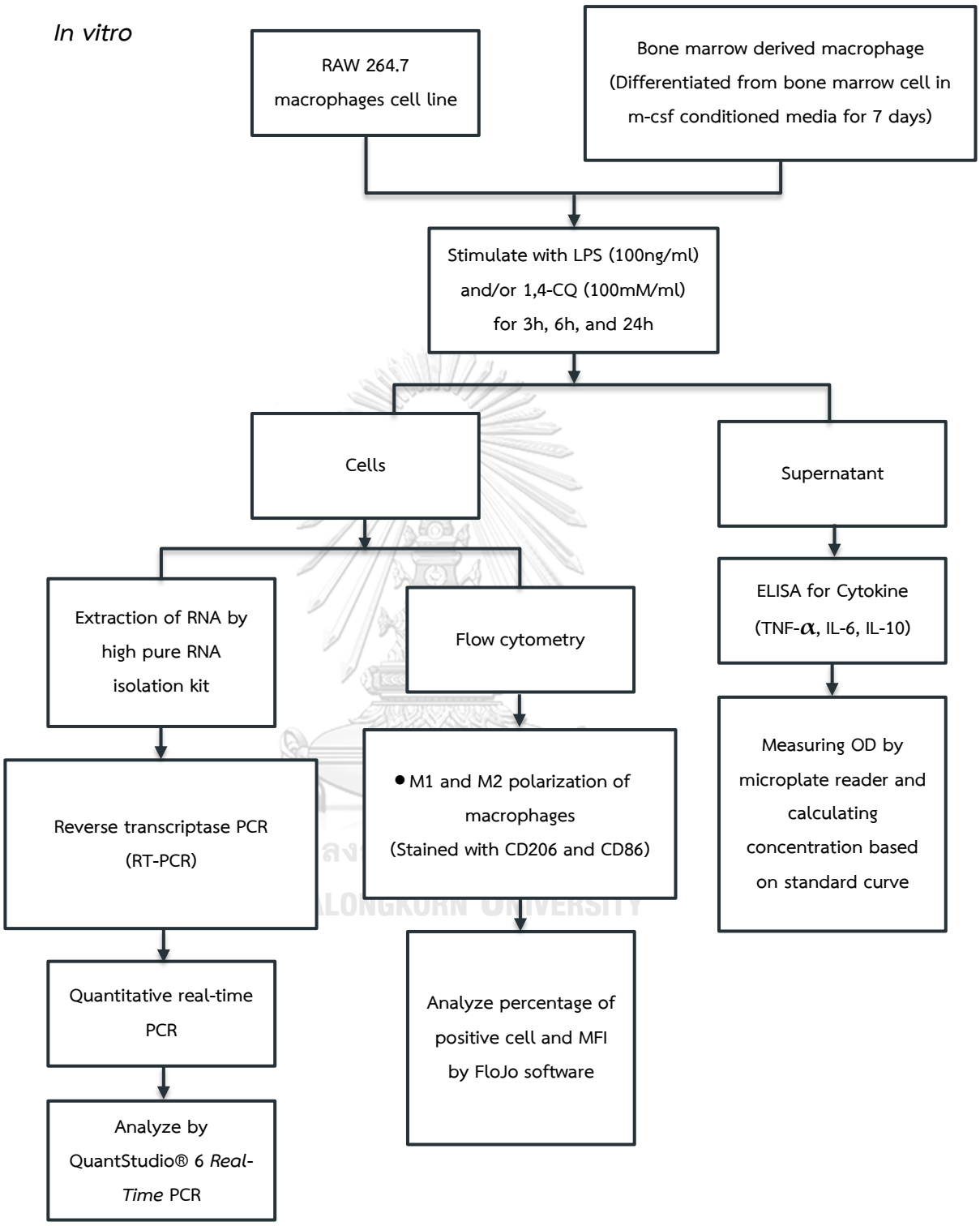
Polycyclic aromatic hydrocarbons with or without LPS induce more inflammation on macrophage cells and in mice FcgRIIb -/- compared to the wild type.

Experimental designs

In vivo



In vitro


RAW 264.7 macrophages cell line
Bone marrow derived macrophage (Differentiated from bone marrow cell in m-csf conditioned media for 7 days)
Stimulate with LPS (100ng/ml) and/or 1,4-CQ (100mM/ml) for 3h, 6h, and 24h
Cells
Supernatant
Extraction of RNA by high pure RNA isolation kit
Flow cytometry
ELISA for Cytokine (TNF-α, IL-6, IL-10)
Reverse transcriptase PCR (RT-PCR)
● M1 and M2 polarization of macrophages (Stained with CD206 and CD86)
Measuring OD by microplate reader and calculating concentration based on standard curve
Quantitative real-time PCR
Analyze percentage of positive cell and MFI by FloJo software
Analyze by QuantStudio® 6 *Real-Time* PCR

CHAHTER V

MATERIALS AND METHODS

Animal model

The animal study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, following the National Institutes of Health (NIH), USA. The lupus FcgRIIb deficient mice on a C57BL/6 background (FcgRIIb-/-) were provided by Dr. Silvia Bolland (NIAID, NIH, Maryland, USA), and wild-type (WT) mice were purchased from Nomura Siam International (Pathumwan, Bangkok, Thailand). C57BL/6 wild type (WT) mice and FcgRIIb -/- mice at 6–10 weeks of age were used. Mice were housed in a selective pathogen-free facility and humidity and temperature-controlled room. C57BL/6 mice and FcgRIIb -/- mice (asymptomatic lupus-prone mice) were received standard mice chow and water during the whole experiment. Mice could adapt to the facility for 1 week. Control animals received an equal amount of water. Lipopolysaccharides (LPS; *Escherichia coli* 026: B6) and/or 1,4-Chrysenequinone (1,4-CQ) were administered via a single intraperitoneal injection (ip) once per day. Either C57BL/6J or FcgRIIb -/- mice divided into 4 groups followed the demonstrated protocol **(Fig 18)** in short-term administration.

1. PBS control group: mice will be received PBS (Control group),
2. PBS/1,4 CQ group; mice received PBS for 24 hours before injection of 1 mM/kg 1,4-CQ,
3. LPS/PBS group; mice received a dose of 4 mg/kg LPS for 24 hours before injection of PBS,
4. LPS/1,4-CQ group; mice received a dose of 4 mg/kg LPS for 24 hours before injected with 1 mM/kg LPS.

For a long term 1,4-CQ administration, once daily ip administration of 1,4-CQ at 1 mM/kg or PBS control, following a publication (12), was performed for 8 weeks with blood collection via tail-vein and/or facial artery in several time-points and through cardiac puncture under isoflurane anesthesia at sacrifice. At sacrifice, kidneys were snap frozen and kept in -80 °C for tissue cytokine analysis and put in Cryogel (Leica Biosystems, Richmond, IL, USA) for fluorescent imaging. The kidneys were washed several times in PBS, weighed, homogenized, and centrifuged for the determination of cytokines in tissue.

Plasma collection

Blood was collected by tail vein and/or facial artery. Blood was collected in sterile tube and centrifuged at 8,000 rpm for 10 minutes at 4°C before the collection in the sterile tube at -80°C. Serum detect Inflammatory cytokine measurement

including interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor-alpha (TNF-α) levels were quantified using Enzyme-Linked Immunosorbent Assay (ELISA) assay. Lupus characteristics were determined by serum anti-dsDNA with the coated Calf-DNA (Invitrogen, Carlsbad, CA, USA) (162) and serum creatinine using QuantiChrom Creatinine-Assay (DICT-500) (BioAssay, Hayward, CA, USA).

Urine collection

The spot urine collection was performed by placing mice in the metabolic cage (Hatteras Instruments, NC, USA) for a few hours (in each time-points) and at 3 h before sacrifice. proteinuria as calculated by spot urine protein creatinine index (UPCI) with an equation; UPCI = urine protein (mg/dL) urine creatinine (mg/dL). Urine protein and creatinine was measured by Bradford Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) and QuantiChrom Creatinine-Assay (DICT-500) (BioAssay), respectively. Absorbent OD was measure by Microplate Reader (Varioskan Flash Multimode ELISA reader, Thermo Fisher Scientific, Waltham, MA USA).

Gut permeability determination

Accordingly, FITC-dextran, a gut non-absorbable molecule, was orally administered to determine gut permeability as previously published (163) by orally administered FITC-dextran (molecular weight 4.4 kDa; Sigma-Aldrich) at 25 mg/mL in

0.25 ml PBS at 3 h before blood collection. Serum FITC-dextran was measured by fluorospectrometry (microplate reader; Thermo Scientific, Wilmington, DE, USA). In addition, serum endotoxin (LPS) was measured as another gut-leakage parameter using the Limulus Amebocyte lysate test (Associates of Cape Cod, East Falmouth, MA, USA) and values of LPS < 0.01 EU/mL were recorded as 0 due to the limitation of the standard curve.

Immunofluorescent imaging

The immunoglobulin deposition in kidneys was visualized by immunofluorescence prepared in Cryogel (Leica Biosystems), stained with goat anti-mouse IgG and DAPI (4′,6-diamidino-2-phenylindole), a blue-fluorescent DNA stain (Alexa Fluor 488; Abcam, Cambridge, MA, USA), then detected and analyzed the fluorescent intensity by ZEISS LSM 800 (Carl Zeiss, Germany). The antibody deposition in lupus mice with high anti-dsDNA indicates immune complex deposition (164).

Ethical issue

This study was approved by the ethical standards of the responsible committee (IRB) on the study of animal at the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no. 023/2563)

Cell differentiation

Macrophages from RAW 264.7 cell line (ATCC®, Manassas, VA20108, USA) were treated with Lipopolysaccharides (LPS; *Escherichia coli* 026: B6) at 100 ng/mL and/or 1,4-Chrysenequinone (1,4-CQ) at 100 nM/mL followed the demonstrated protocol **(Fig 9)** in a 5% CO_2 tissue culture incubator at 37°C. Cells were fed with DMEM (Dulbecco'Modified Eagle Medium) supplemented with 5% fetal bovine serum and 1% PenStrep, to promote differentiation into macrophages. Cell were harvested with cold PBS. RAW 264.7 cell line was collected by centrifugation at 2,600 rpm at 4°c, 5 min and supernatant were stored at -80°C.

Bone marrow derived macrophage (BMM) preparation

Mouse bone marrow was flushed from the femurs and tibias of mice at 6–10 weeks of age. The bone marrow cells were plated in petri dishes in DMEM-high glucose with 10% *(v/v)* Fetal Bovine Serum (FBS), 1% *(v/v)* HEPES, 1% *(v/v)* sodium pyruvate and 1.3% *(v/v)* PenStrep. Cells were fed on day 4 in completed DMEM with horse serum and macrophage colony-stimulating factor (M-CSF). On day 7, cells were removed from petri dishes and cultured on tissue culture dishes in completed DMEM. The 1 x 10^6 cells/well in 6-well plates in total 1 ml of completed DMEM under 5% CO_2 at 37°c for 24 hours. After the stimulation with LPS and/or 1,4-CQ followed the demonstrated protocol **(Fig 12)**. Cells were harvested with cold PBS.

Bone marrow cells were collected by centrifugation at 2,600 rpm at 4°C, 5 min and supernatant were stored at -80°C. Anti-F4/80 and anti-CD11c antibody staining by BD LSR II Flow Cytometry were used for characterization of the macrophage phenotype.

Quantitative real-time PCR

The RAN isolation from macrophage cells were carried out using a high pure RNA isolation kit, the measurement of concentration of RNA by using NanoDrop spectrophotometer were performed. cDNA was prepared using reverse transcriptase. Gene expression was analyzed by 2XPowerUp™SYBR™Green Master Mix according to the manufacturer's instructions and performed using QuantStudio® 6 *Real-Time* PCR system. All primers were purchased from Integrated DNA Technologies, Inc. (IDT), The list of primers is shown in **Table 1**. Cycling conditions of real-time PCR were 95℃ for 10 min; 40 cycles of 95℃ for 15s, 60℃ for 1min. Negative controls are concomitantly run to confirm that the samples are not cross-contaminated. ß-actin was used as a reference gene, and relative quantification analysis is determined via the $2^{-\Delta\Delta Ct}$ method.

Name	Forward primer	Reverse primer
Arginase-1 (Arg-1)	5′-CTTGGCTTGCTTCGGAACTC-3′	5′-GGAGAAGGCGTTTGCTTAGTT-3′
Aryl hydrocarbon receptor (Ahr)	5′-GACCACTTAGAGCACCACTA-3′	5′-AGAACTTCAATCAGACATACACAA-3′
Fc gamma receptor IIb (FcgRIIb)	5′-TTCTCAAGCATCCCGAAGCC-3′	5′-TTCCCAATGCCAAGGGAGAC-3′
Inducible nitric oxide synthase (iNOS)	5′-ACCCACATCTGGCAGAATGAG-3′	5′-AGCCATGACCTTTCGCATTAG-3′
Interleukin-1β (IL-1β)	5′-GAAATGCCACCTTTTGACAGTG-3′	5′-TGGATGCTCTCATCAGGACAG-3′
Nuclear factor-κB (NF-κB RelA)	5′-CTTCCTCAGCCATGGTACCTCT -3′	5′-CAAGTCTTCATCAGCATCAAACTG-3′
Resistin-like molecule-α (FIZZ-1)	5′-GCCAGGTCCTGGAACCTTTC-3′	5′-GGAGCAGGGAGATGCAGATGA-3′
Toll like receptor 4 (TLR-4)	5′-GGCAGCAGGTGGAATTGTAT-3′	5′-AGGCCCCAGAGTTTTGTTCT-3′
Transforming Growth Factor-β (TGF-β)	5′-CAGAGCTGCGCTTGCAGAG-3′	5′-GTCAGCAGCCGGTTACCAAG-3′
β-actin	5′-CGGTTCCGATGCCCTGAGGCTCTT-3′	5′-CGTCACACTTCATGATGGAATTGA-3′

Table 1. List of Primers in the study are demonstrated**.**

Flow cytometry

BMM suspended in PBS at a concentration of 1 × 10^6 cells/mL were stained for macrophage polarization by fluorescein isothiocyanate (FITC)-labeled CD206 (1 μL/well) and allophycocyanin (APC)-labeled CD86 antibodies (1 μL/well) (BD Biosciences, San Jose, CA, USA) for the M2 and M1 macrophage polarization, respectively. Then the samples were washed with FACS flow buffer, PBS supplemented with 1% *(v/v)* FBS and 0. 05% NaN3 and processed in a BD LSR II Flow Cytometry (BD Biosciences) using the FloJo software (Tree Star Inc., Ashland, OR, USA).

Enzyme-linked immunosorbent assay (ELISA)

Cell culture medium supernatant and mouse serum samples were stored at −80 ℃ and thawed at room temperature before assay. Subsequent steps were performed according to the manufacturer's instructions. Cytokine levels were measured by Microplate Reader.

Statistical analysis

To detect differences in gene expression, all expression data were analyzed by the Statistical Package for Social Sciences software (SPSS 22.0, SPSS Inc., IL, USA) and Graph Pad Prism version 7.0 software (La Jolla, CA, USA). The results were presented as mean ± standard deviation (S.D). The Mann-Whitney unpaired t-test was carried out to determine the differences in the expression between groups or one-way analysis of variance (ANOVA) with Tukey's comparison test for the analysis of experiment with two group or more than two groups, respectively. $P < 0.05$ was considered as statistically significant.

CHAPTER VI

RESULT

The pre-treatment with LPS amplified expression of aryl hydrocarbon receptor (Ahr) resulted in the more profound responses of FcgRIIb-/- macrophages than WT cells. Indeed, the administration of PAHs activator in FcgRIIb-/- mice induced the more severe inflammation and activated lupus characteristics, implying a possible prominent adverse effect of PM2.5 in lupus.

Pre-treatment with LPS enhanced macrophage responses toward PAHs activator, but not vice versa, in the macrophage cell-line

Due to the predominant foreign body recognition property of macrophages, RAW264.7 cell-line was used for an initial exploration on PAHs activation. Surprisingly, PAHs activation alone by 1,4-CQ (N/1,4-CQ) did not induce any inflammatory responses in macrophages as determined by supernatant cytokines and the expression of several genes **(Fig 10A-F and Fig 11A-D).** Meanwhile LPS, a potent inflammatory activator, without the pretreatment (N/LPS) induced the strong macrophage responses but did not different from LPS stimulation after 1,4-CQ pretreatment (1,4-CQ/LPS) **(Fig 10A-F and Fig 11A-D).**

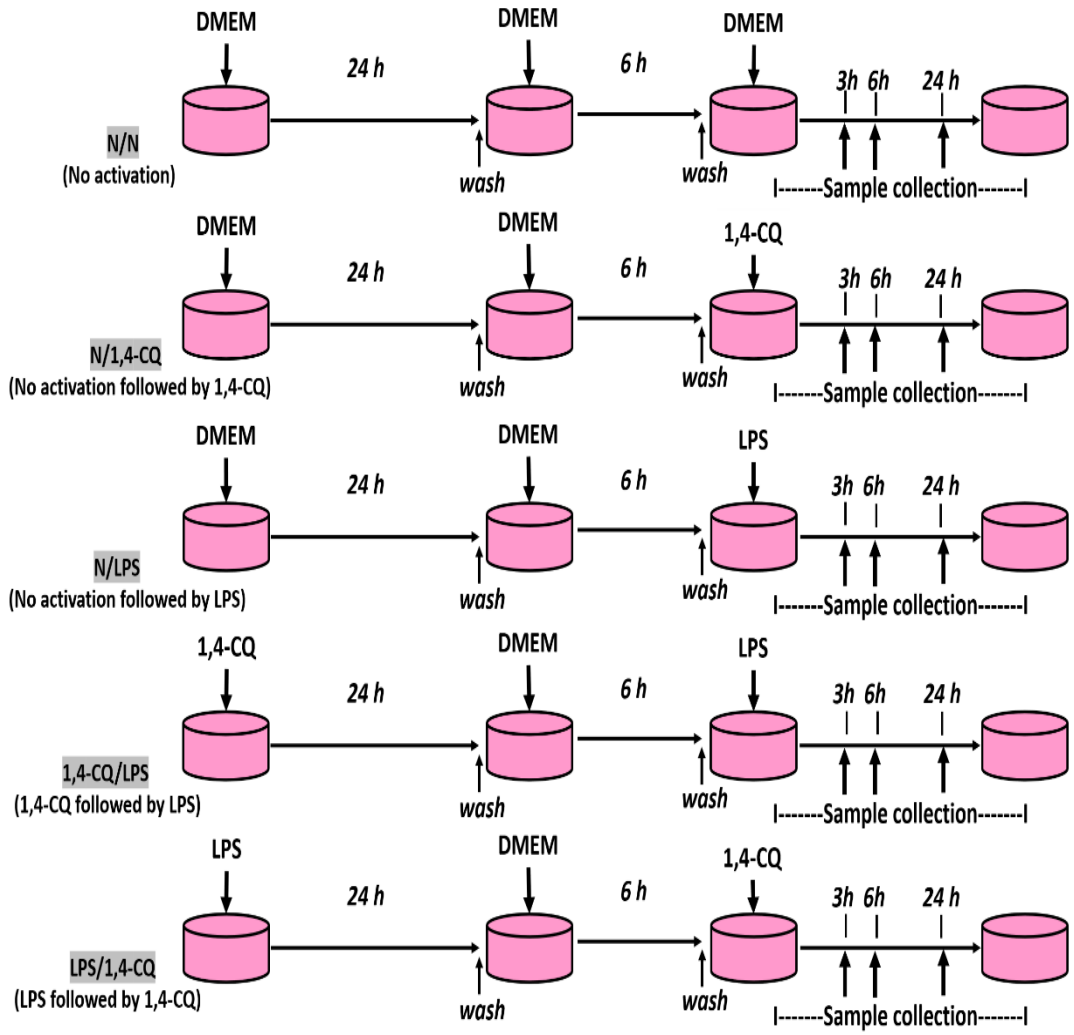


Figure 9. Schema of the in vitro experiments in RAW 267.4 cell line is demonstrated (details in method).

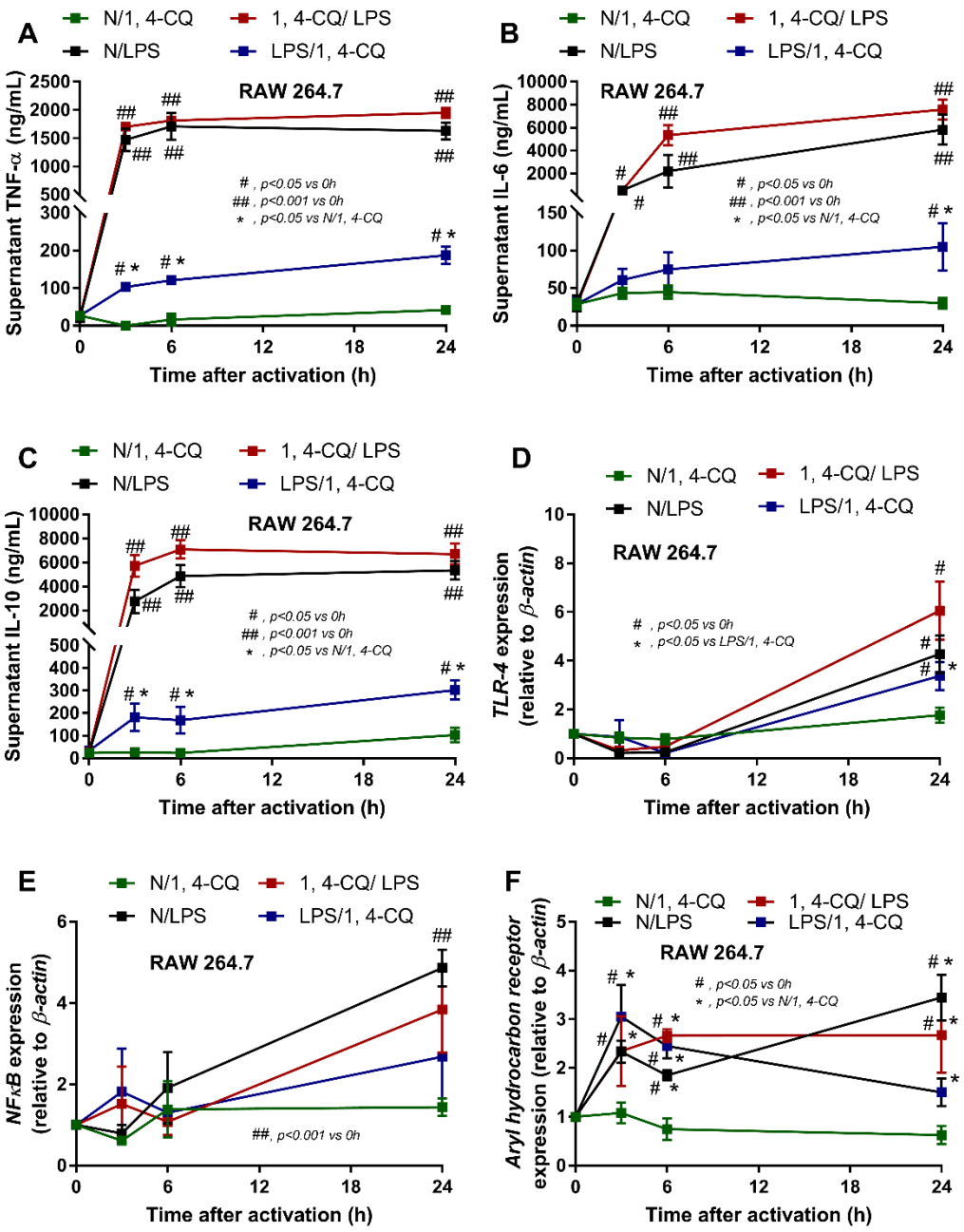


Figure 10. Characteristic of the responses in RAW246.7, a macrophage cell-line, against a single activation by Polycyclic Aromatic Hydrocarbons; 1,4-chrysenequinone (N/1,4-CQ), or a positive-control stimulator, lipopolysaccharide (N/LPS), and the activation with the pre-treatment protocol (1,4-CQ/LPS and LPS/1,4-CQ) as demonstrated by supernatant cytokines (A-C) and signalling mediators (*TLR-4, NF-κB, aryl hydrocarbon receptor)* (D-F) are demonstrated.

Meanwhile, LPS pre-treatment before 1,4-CQ (LPS/1,4-CQ), when compared with PAHs activation alone (N/1,4-CQ), enhanced several inflammatory markers, including supernatant cytokines and gene expression of *TLR-4*, *iNOS* and *IL-1β* **(Fig 10A-F and Fig 11A-D).** Of note, 6 h of control media incubation after 24 h LPS stimulation **(Fig 9)** set a baseline of all markers as indicated in the 0 time-point in LPS/1,4-CQ group **(Fig 10A-F and Fig 11A-D).** Interestingly, an enhanced *Ahr* expression after LPS stimulation, as indicated in N/LPS group, made it easier for the Ahr re-activation as demonstrated by high *Ahr* expression as early as 3 h after 1,4-CQ stimulation in LPS/1,4-CQ group **(Fig 10F)** and perhaps responsible for a more sensitivity towards the effects of Ahr ligands.

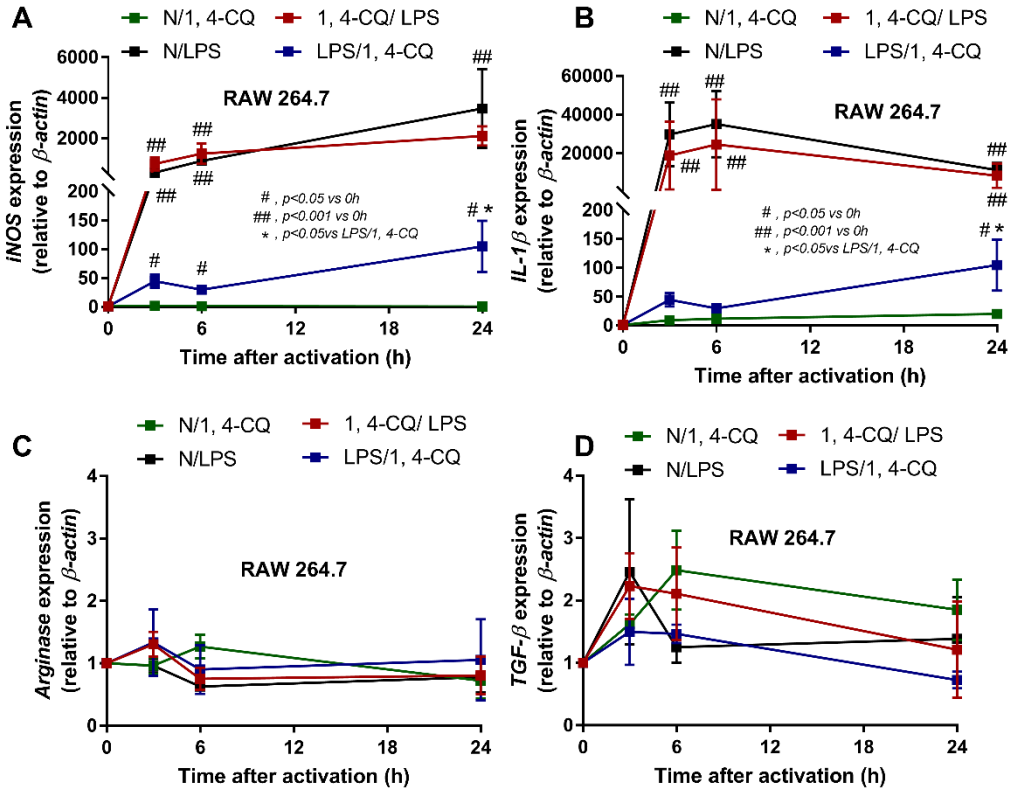


Figure 11. Characteristic of the responses in RAW246.7, a macrophage cell-line, against a single activation by Polycyclic Aromatic Hydrocarbons; 1,4-chrysenequinone (N/1,4-CQ), or a positive-control stimulator, lipopolysaccharide (N/LPS), and the activation with the pre-treatment protocol (1,4-CQ/LPS and LPS/1,4-CQ) as demonstrated by macrophage polarization–genes *(iNOS, IL-1β, arginase, TGF-β)* (A-D) are demonstrated.

Pre-treatment with LPS before PAHs activation in the FcgRIIb-/- macrophages induced the more severe inflammation than WT cells

Because of the hyper-responsiveness of FcgRIIb-/- macrophages towards several stimulations (165, 166), PAHs activation was tested in FcgRIIb-/- and WT cells. With LPS stimulation alone (N/LPS), FcgRIIb-/- macrophages demonstrated the potent inflammatory responses than WT cells as indicated by the higher supernatant cytokines and the expression of several genes (at least in one time-point) including *TLR-4*, *NF-κB* and *Ahr* **(Fig 13A-C and Fig 14A-C, left graphs).** Meanwhile, there was no response in both cells after the stimulation by PAHs activator alone (N/1,4-CQ) **(Fig 13A-C and Fig 14A-C, left graphs).** In parallel, pretreatment of 1,4-CQ before LPS stimulation (1,4-CQ/LPS) did not significantly altered macrophage responses compared with N/LPS stimulation in both mouse strains despite the more predominant responses in FcgRIIb-/- cells over WT cells **(Fig 13A-C and Fig 14A-C, left graphs).** On the other hand, LPS pretreatment before PAHs stimulation (LPS/1,4-CQ) in FcgRIIb-/- macrophages induced the higher responses than N/1,4-CQ

as indicated by supernatant cytokines and gene expression of *Ahr* which were higher in FcgRIIb-/- macrophages than W T cells **(Fig 13A-C and Fig 14A-C, right graphs).**

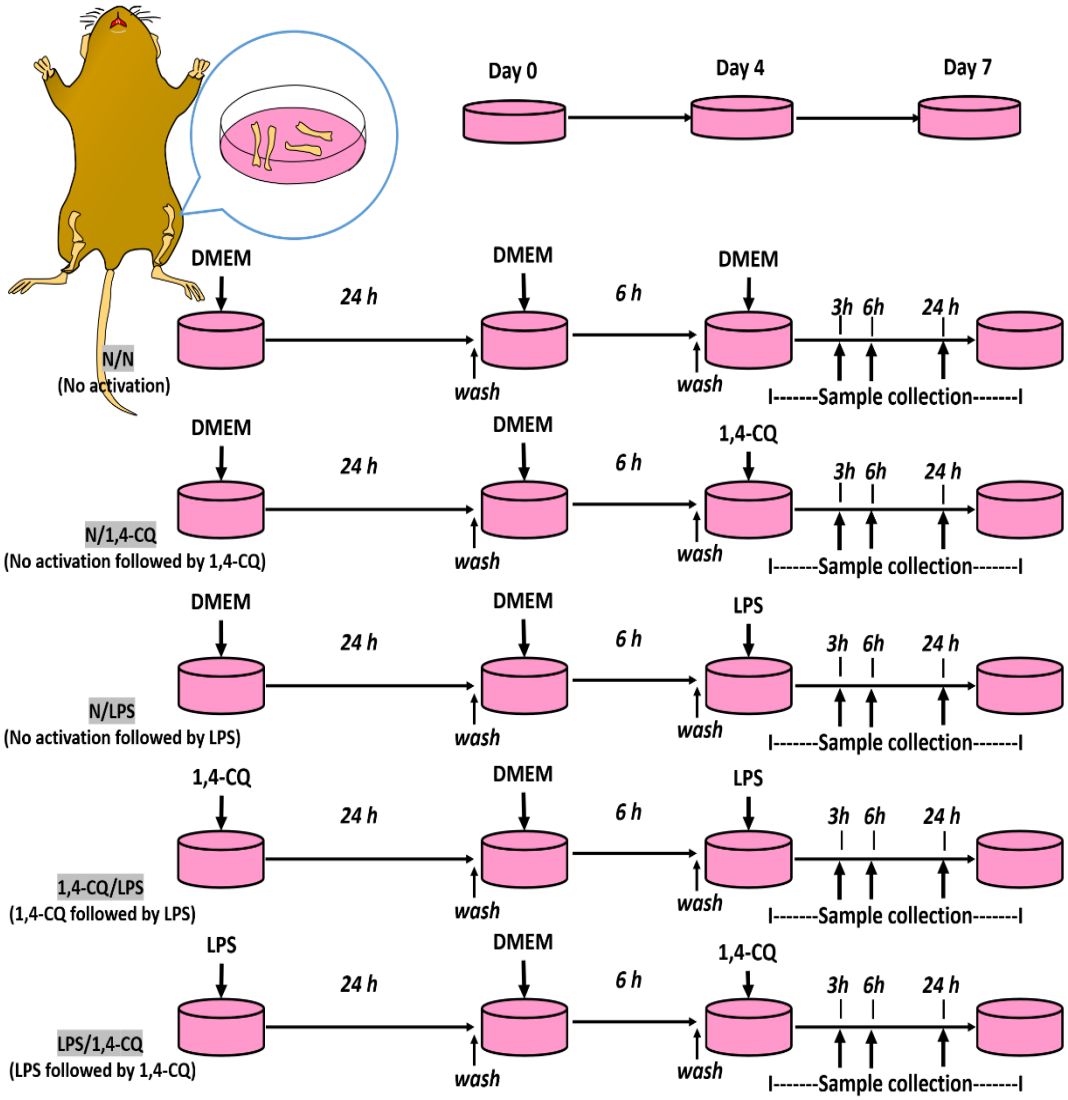


Figure 12. Schema of the *in vitro* experiments in bone marrow-derived macrophages (BMM) are demonstrated (details in method).

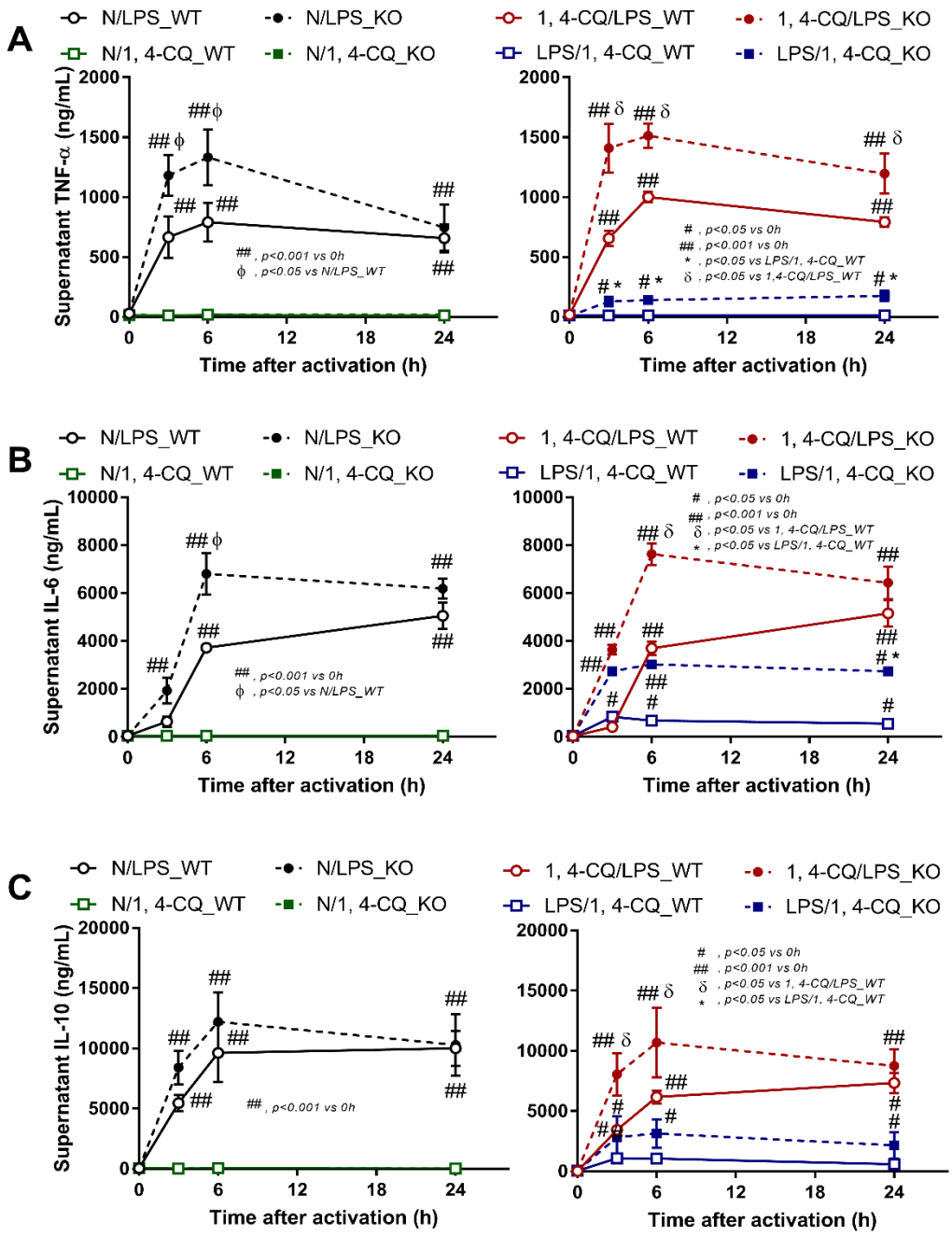


Figure 13. Characteristics of the responses in FcgRIIb-/- lupus macrophages (KO) and wild-type cells (WT) after a single activation by Polycyclic Aromatic Hydrocarbons; 1,4-chrysenequinone (N/1,4-CQ), or a positive-control stimulator, lipopolysaccharide (N/LPS) (left side of each graph)

and the activation after the pre-treatment protocol (1,4-CQ/LPS and LPS/1,4-CQ) (right side of each graph) as determined by supernatant cytokines (A-C) are demonstrated.

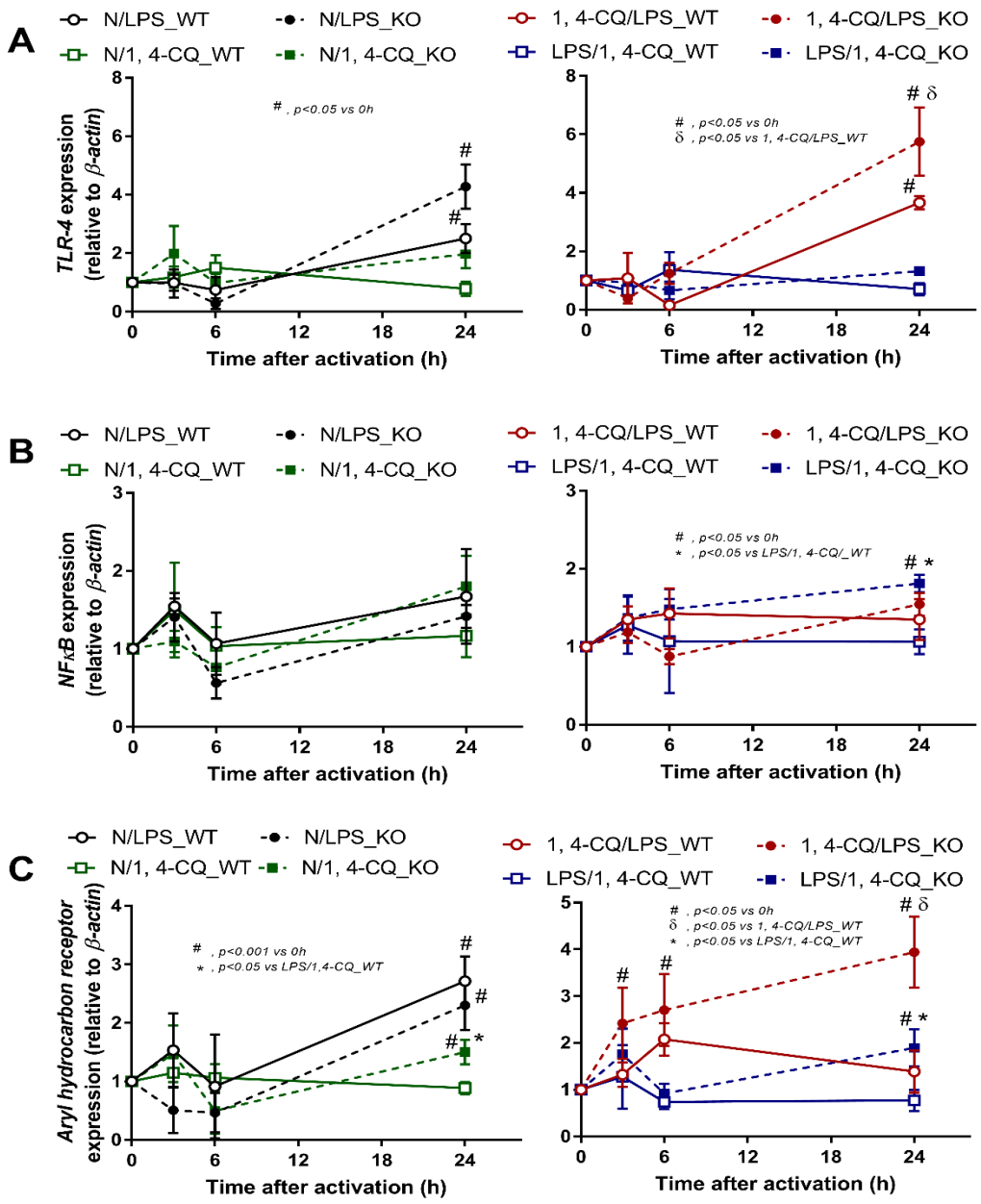


Figure 14. Characteristics of the responses in FcgRIIb-/- lupus macrophages (KO) and wild-type cells (WT) after a single activation by Polycyclic Aromatic Hydrocarbons; 1,4-chrysenequinone (N/1,4-CQ), or a positive-control stimulator, lipopolysaccharide (N/LPS) (left side of each graph)

and the activation after the pre-treatment protocol (1,4-CQ/LPS and LPS/1,4-CQ) (right side of each graph) as determined by signalling mediators (*TLR-4, NF-κB, aryl hydrocarbon receptor*) (A-C) are demonstrated.

Due to the prominent inflammatory responses of M1-polarized macrophages (167) and the cross-talk between FcgRs and TLR-4 (168), macrophage polarization and FcgRs were explored. Accordingly, in N/LPS and 1,4-CQ/LPS, there was a similar M1 macrophage polarization **(Fig 15A-D and Fig 16A-F).** Meanwhile, there was a non-responsiveness after N/1,4-CQ stimulation in macrophages from both strains but prominent M1 macrophage polarization (*iNOS*, *IL-1β* and CD86) compared with WT cells **(Fig 15A-D and Figure 16A-F).** Notably, the absence in the expression of inhibitory-*FcgRIIb* **(Fig 17)** might be associated with the hyper-responsiveness in FcgRIIb-/- macrophages.

Prominent inflammatory activation and lupus exacerbation after the activation of polycyclic aromatic hydrocarbons in FcgRIIb-/- lupus mice

It is well-known that lupus activity exacerbation by inflammation (169, 170). Hence, 1,4-CQ was intraperitoneally administered in 8-week-old FcgRIIb-/- and WT mice in a short-term administration **(Fig 18 schema)** to explore the inflammatory activity. As such, PAHs activator alone (PBS/1,4-CQ) did not induce inflammatory cytokines in both FcgRIIb-/- and WT mice **Fig 18A-C).** Meanwhile inflammation in FcgRIIb-/- mice after 24 h of LPS activation (LPS/PBS) was slightly higher than WT cells

as indicated by TNF-α (at 0 h and 2 h time-point; equal to 24 h and 26 h post-LPS) and IL-10 (at 2 h time point; equal to 26 h post-LPS) in LPS/PBS group **Fig 18A-C).** Nevertheless, LPS pre-treatment in mice enhanced the responses of PAHs activator predominantly in FcgRIIb-/- mice than WT at 2 h and 6 h in LPS/1,4-CQ group despite the non-responsiveness in PBS/1,4-CQ group **Fig 18A-C).**

Although there was a limited response of FcgRIIb-/- mice in a single stimulation by PAHs activator, a long-term administration might be different, considering the vulnerability against several stimuli in patients with lupus (24). Accordingly, the 8 weeks once daily 1,4-CQ intraperitoneal injection induced anti-dsDNA, proteinuria, and systemic inflammation (but not serum creatinine) only in FcgRIIb-/- mice (**Fig 19A-F).** In addition, the systemic inflammation from PAHs activation in FcgRIIb-/- lupus mice induced gut leakage, and endotoxemia **(Fig 19G-M)** that possibly worsening lupus conditions. Notably, PBS control administered FcgRIIb-/- mice did not demonstrated any lupus characteristics despite the increased age of the lupus mice **(Fig 19A-M).**

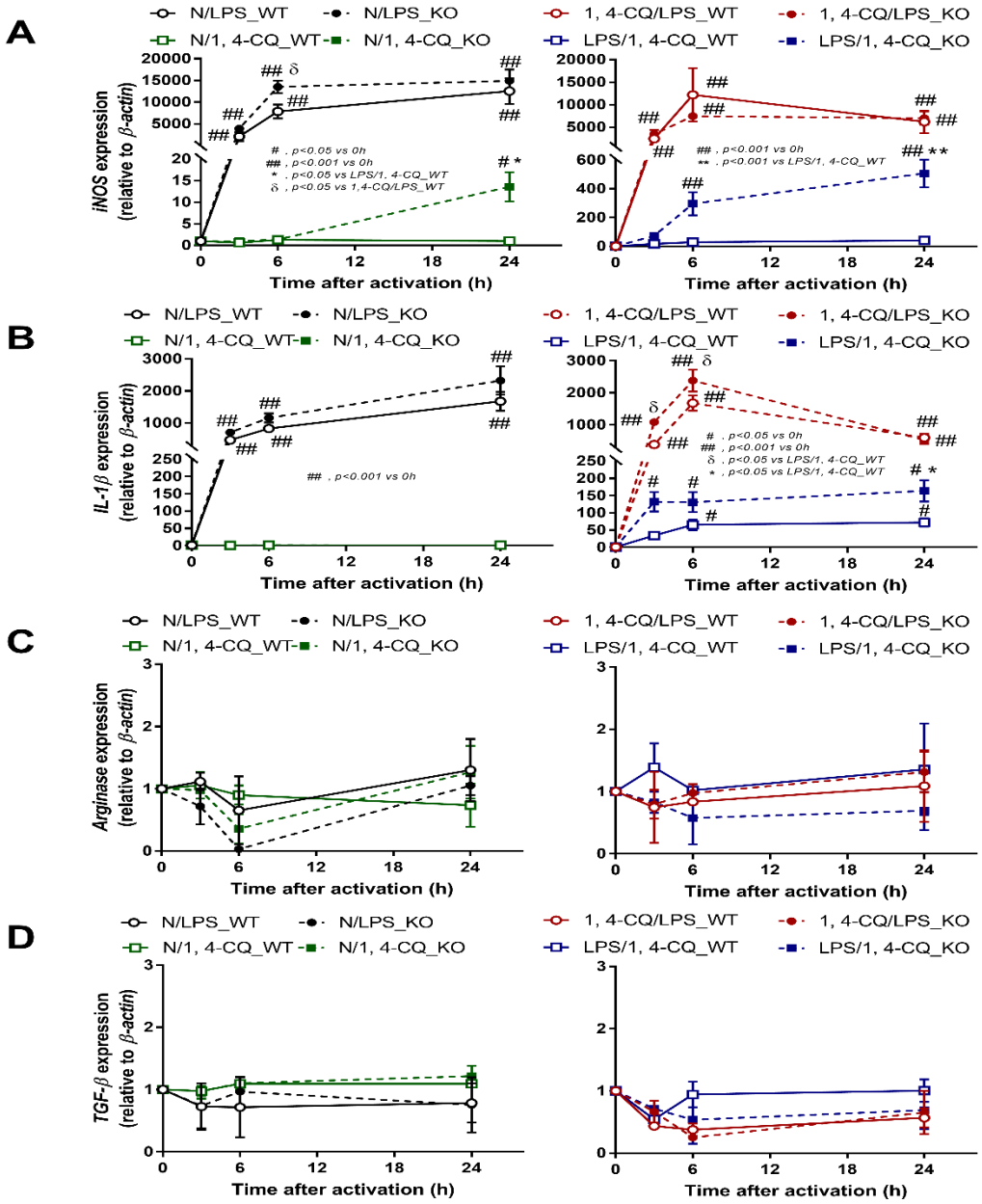


Figure 15. Characteristics of the responses in FcgRIIb-/- lupus macrophages (KO) and wild-type cells (WT) after a single activation by Polycyclic Aromatic Hydrocarbons; 1,4-chrysenequinone (N/1,4-CQ), or a positive-control stimulator, lipopolysaccharide (N/LPS) and the activation after the pre-treatment protocol (1,4-CQ/LPS and LPS/1,4-CQ) as determined by the expression of macrophage polarization genes *(iNOS, IL-1β, arginase, TGF-β)* (A-D). Independent triplicate experiments were performed.

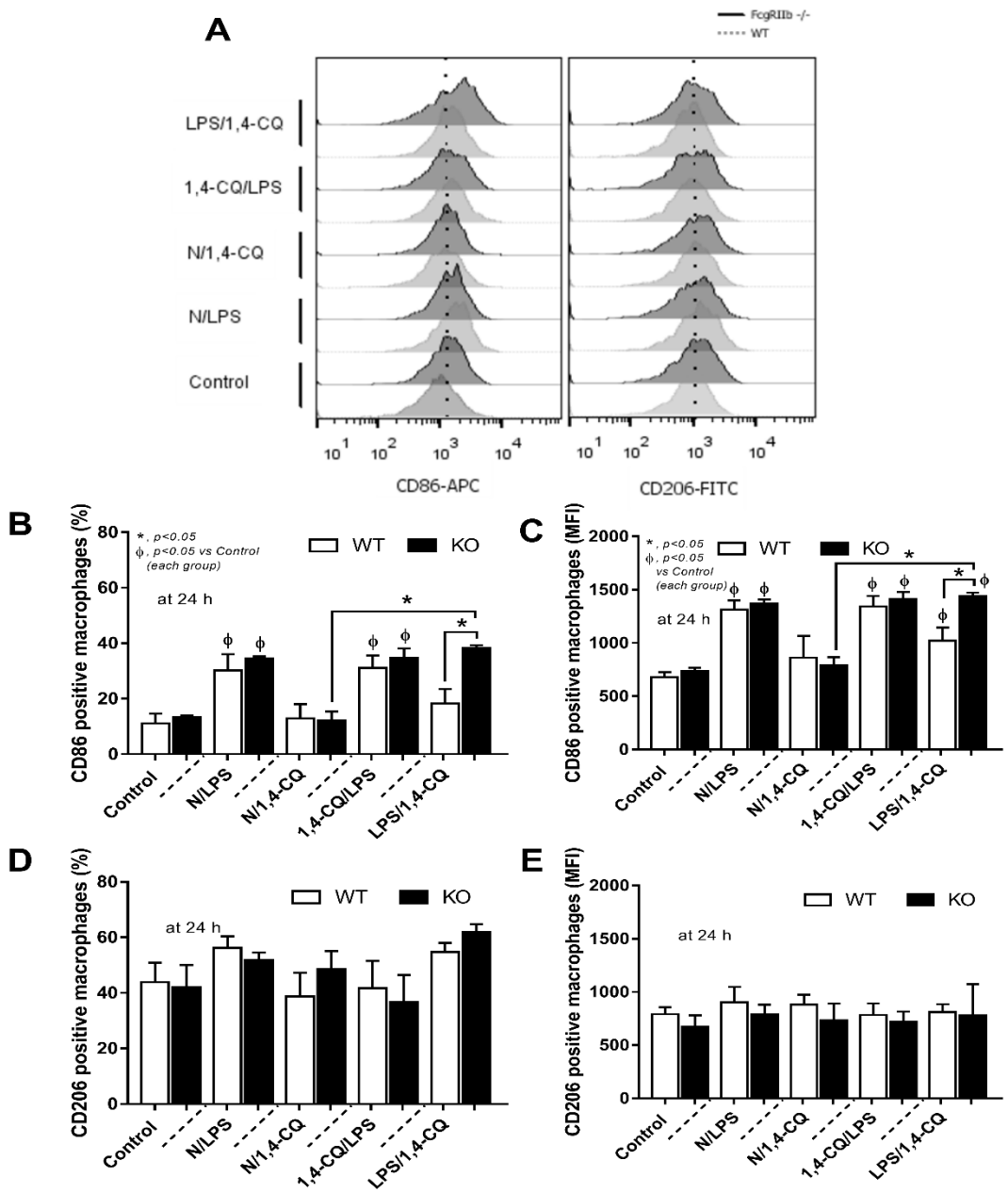


Figure 16. The representative pictures of flow cytometry analysis of CD86 (APC) and CD206 (FIT-C), a marker of M1 and M2 macrophage polarization, respectively, at 24 h post-stimulation by Polycyclic Aromatic Hydrocarbons; 1,4-chrysenequinone (N/1,4-CQ), or a positive-control stimulator, lipopolysaccharide (N/LPS) and the activation after the pre-treatment protocol (1,4-CQ/LPS and LPS/1,4-CQ) or control media alone (control) are demonstrated.

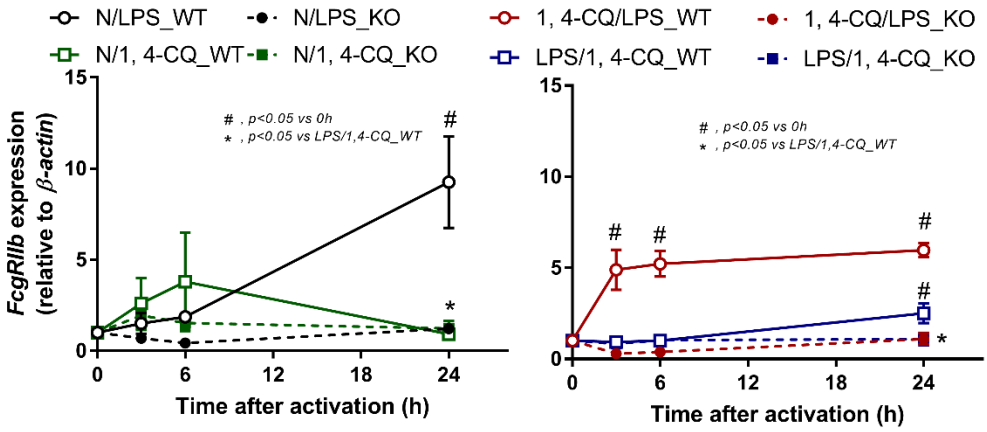


Figure 17. Characteristics of the responses in FcgRIIb-/- lupus macrophages (KO) and wild-type cells (WT) after a single activation by Polycyclic Aromatic Hydrocarbons; 1,4-chrysenequinone (N/1,4-CQ), or a positive-control stimulator, lipopolysaccharide (N/LPS) and the activation after the pre-treatment protocol (1,4-CQ/LPS and LPS/1,4-CQ) as determined by the expression of Fc gamma RIIb receptor (FcgRIIb) after these stimulations are also demonstrated.

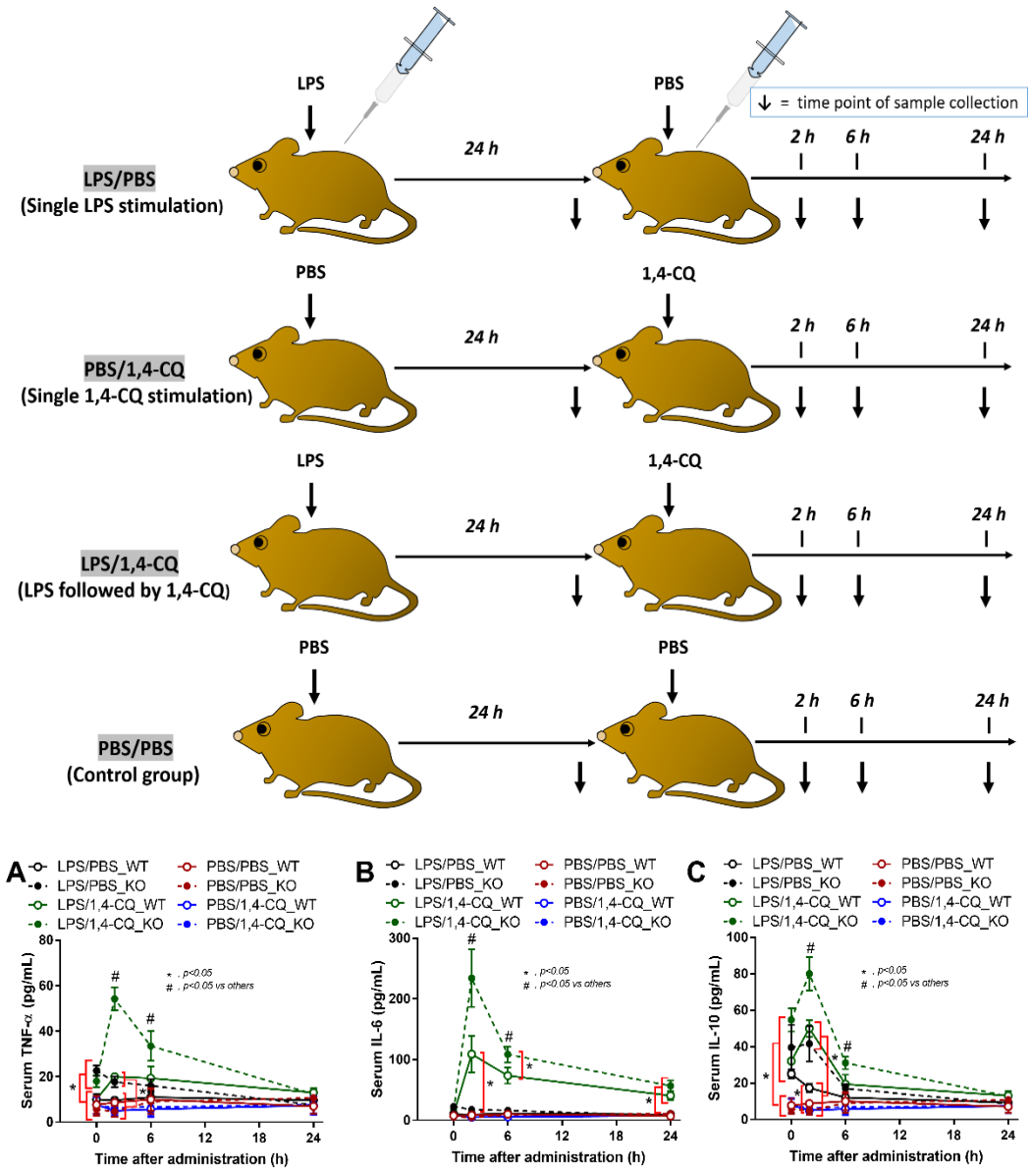


Figure 18. Schema of the short-term experiments by the pre-treatment with lipopolysaccharide (LPS) or control phosphate buffer solution (PBS) at 24 h before an administration by a Polycyclic Aromatic Hydrocarbons; 1,4-chrysenequinone (1,4-CQ), or PBS is demonstrated (upper part of figure). Additionally, the characteristics of FcgRIIb-/- lupus mice (KO) and wild-type mice (WT) after the induction by control PBS alone (PBS/PBS), 1,4-CQ alone (PBS/1,4-CQ), LPS pre-treatment following by PBS (LPS/PBS) or 1,4-CQ (LPS/1,4-CQ) as determined by serum cytokines (A-C) are demonstrated.

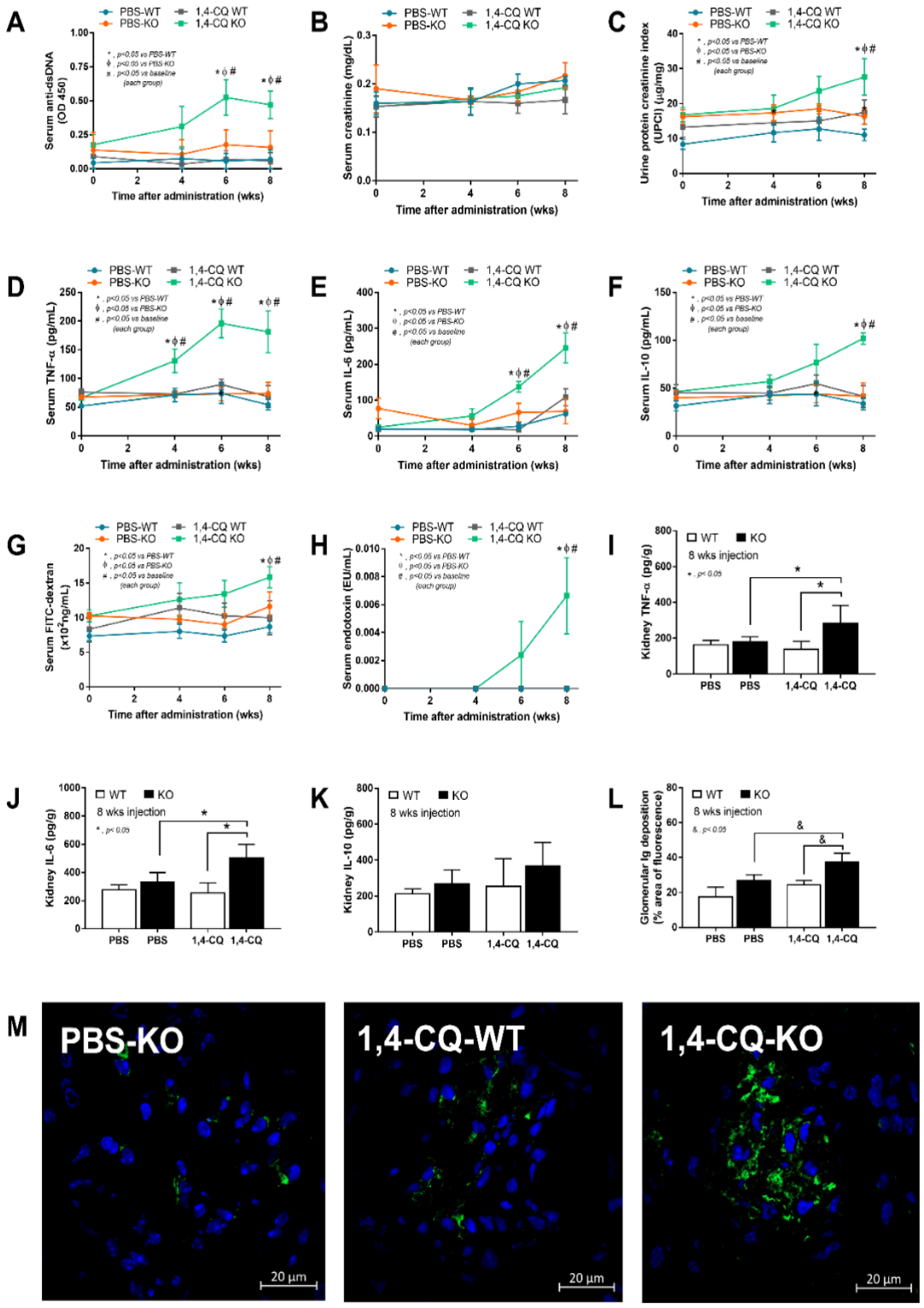


Figure 19. The characteristics of FcgRIIb-/- lupus mice (KO) and wild-type mice (WT) after the once daily 8-wk-administration by control PBS or an aryl hydrocarbon receptor activator, 1,4-

chrysenequinone (1,4-CQ), as determined by lupus characteristics (anti-dsDNA, serum creatinine and urine protein creatinine index) (A-C), serum cytokines (D-F), gut leakage (FITC-dextran assay and endotoxemia) (G, H), cytokines from kidney tissue (I-K), glomerular immunoglobulin deposition (L) and the representative immunofluorescence pictures from glomeruli after administration (M) are demonstrated. The original magnification of the glomeruli is 200x, green and blue colour demonstrated mouse IgG and intestinal nuclei, respectively. The picture of PBS-administered wild-type control mice (PBS-WT) is not shown due to the similarity to PBS-KO group.

CHAPTER VII

Discussion

Here, LPS increased *Ahr* expression that enhanced the responses toward PAHs, a representative of PM2.5 stimulation, more prominently in FcgRIIb-/- lupus macrophages when compared with WT cells. The long-term PAHs administration in asymptomatic lupus FcgRIIb-/- mice induced lupus activity as indicated by serum anti-dsDNA and glomerular immune deposition suggested a possible adverse effect of PM2.5 on lupus.

The enhanced macrophage responses against polycyclic aromatic hydrocarbons activator through aryl hydrocarbon receptor by a pre-conditioning immune activation, an impact of inflammation to the air pollution

The sequential stimulation beginning with LPS following by PAHs activator enhanced the inflammatory responses when compared with PAHs activator alone implied the effect of inflammation before the stimulation by air pollution. Despite the non-responsiveness in N/1,4-CQ, the inflammatory markers increased as early as 3-6 h in LPS/1,4-CQ group including cytokines, inflammatory mediators (*TLR-4*, *Ahr* and *NF-κB*), M1 macrophage polarization (*iNOS*, *IL-1β* and CD86), and inhibitory-*FcgRIIb*. The crosstalk between TLR-4 and activating-FcgRs (168), without inhibitory

signaling, and the shift of balance toward activating-FcgRs (171) might be responsible for the enhanced responses toward Ahr activator by LPS pre-treatment in RAW246.7 cells. In contrast, the PAHs activator pre-conditioning before LPS stimulation did not alter LPS responses implied a non-inflammatory response against PAHs activation alone, highlighted an importance of other components in PM2.5 as previously mentioned (18, 19, 84). Although the investigation using isolated PM2.5 is necessary, our data supported the importance of inflammation before the stimulation by air pollution (172-174).

Prominent inflammatory responses in FcgRIIb-/- macrophages over the wild-type cells, an inhibitory effect of FcgRIIb

The hyper-immune responsiveness related to a defect in negative-FcgRIIb signaling in FcgRIIb-/- macrophages of the lupus model is demonstrated (129, 165). Interestingly, PAHs activation alone did not induce inflammatory responses in either FcgRIIb-/- and WT macrophages but the LPS pretreatment predominantly enhanced the responses of PAHs activator more predominantly in FcgRIIb-/- macrophages than WT cells. Despite LPS hyper-responsiveness of FcgRIIb-/- macrophages (165), the 6 h culture-media washing-out procedure **(Fig 12)** reduced all parameters into the baseline. Interestingly, PAHs activation after LPS (LPS/1,4-CQ) in FcgRIIb-/- macrophages demonstrated the more severe inflammatory markers including *TLR-4*,

and *Ahr*, but not the inhibitory-*FcgRIIb*. Because of i) the potent TLR-4 activation of LPS (175), ii) the TLR-4 and FcgRs crosstalk (168), iii) the enhanced LPS effect by Ahr activation through NF-κB subunit (176) and iv) the inflammation-induced apoptosis (177-179), the increase in activating-FcgRs (without an inhibitory-FcgRIIb), TLR-4 and Ahr in macrophages might be responsible for the hyper-responsiveness of FcgRIIb-/- cells over the WT (168, 176, 180) as concluded in the previous publications (166, 181). The enhanced Ahr in LPS-preprogrammed macrophages made it easier for Ahr activator for the inflammatory stimulation and the inflammation is stronger in FcgRIIb-/- macrophages due to the lack of the inhibitory signaling. Because both inflammatory cytokines and cell apoptosis are the exacerbation factors in lupus, the Ahr activation might exacerbate lupus activity (182).

The enhanced lupus activity through the aryl hydrocarbon receptor, a possible impact of air pollution in lupus

Accordingly, an PAHs activator was administered in FcgRIIb-/- and WT mice in a short- and long-term administration. In a short-term administration, PAHs activation alone did not induce inflammatory cytokines in similar to the *in vitro* experiments while LPS pretreatment before PAHs activation (LPS/1,4-CQ) induced the higher cytokines predominantly in FcgRIIb-/- when compared with WT mice. Notably, there was the slightly higher inflammation in FcgRIIb-/- mice over WT mice before PAHs

stimulation (after LPS) as indicated in the 0 h time-point in LPS/PBS (24 h post-LPS). Thus, 1,4-CQ administration in LPS/1,4-CQ protocol was a stimulation upon an active inflammation implying that the exposure to air pollution during a slight inflammation exert the higher inflammatory responses. These data also support the enhanced adverse effects of air-pollution in patients with active respiratory inflammation (172-174).

Although the short-term PAHs activation alone did not induce inflammation, the long-term PAHs administration increased serum cytokines only in FcgRIIb-/- lupus mice, but not WT, as early as 4 weeks of the administration and induced lupus activity as indicated by anti-dsDNA, kidney immune deposition and cytokines in kidney tissue which possibly were an early signs of lupus nephritis. These data support the exacerbation of lupus activity through the active inflammation (169, 170). Furthermore, the level of PAHs-activated inflammation was high enough to cause gut leakage with mild endotoxemia at 8 weeks of the administration that might further enhanced the more severe inflammation (164). Endotoxin, also refer to as LPS, is a major cell-wall component of Gram-negative bacteria which are the predominant gut-microbiota with a potent inflammatory activation property than the host antigens (183). Without the inhibitory FcgRIIb, TLR-4 is possibly cross-linked with only the activating FcgRs that causes hyper-immune responses in FcgRIIb-/- mice (166, 181). As such, these data support PAHs-induced pro-inflammation after a long-term stimulation (184, 185) despite a possible anti-inflammation of PAHs activator in

several short-term stimulations (186). Hence, the pre-conditioning, the duration of stimulation and the potency of PAHs stimulator might be associated with the different responses. Indeed, the co-stimulation of short-term LPS with PAHs activator reduced inflammation (12, 14) while, in here, the short-term PAHs activation after LPS in our data enhanced inflammation. Although more studies in this topic are necessary for a solid conclusion, a proof of concept in a possible more severe adverse effect from air pollution in patients with lupus is demonstrated. Hence, a proper protection against air pollution for these patients should be considered.

In conclusion, our data supported the prominent hyperinflammatory responses to PAHs stimulation in FcgRIIb-/- lupus mice, over WT mice, especially with LPS-pretreatment. Because PAHs is a major component of PM2.5 that activate immune responses through Ahr, the prominent inflammatory effect of PAHs activator in FcgRIIb-/- macrophages and in mice when compared with WT groups indicated a possible more severe adverse effect of air pollution in patients with lupus. More studies are interesting.

APPENDIX A

MATERIALS AND EQULPMENT

1. 6 wells plate (Cell Culture plate)	USA
2. 96 wells flat bottom plate	USA
3. 96 wells PCR microplate	USA
4. BD LSR II Flow Cytometry	USA
5. Biosafety Cabinet Class II NU-4oo-600E	USA
6. Centrifuge 5415R	USA
7. Centrifuge U-32R	Germany
8. CO_2 incubator NU-5500E	USA
9. Decimal balance XT-220A	Switzerland
10. Filter pipet tip 10, 200, 1000 µL	USA
11. Micropipette 1, 10, 200, 1000 µL	Germany
12. NanoDrop 1000	USA
13. Pipet tip 10, 200, 1000 µL	USA
14. ProFlex PCR system	USA
15. QuantStudio® 6 *Real-Time* PCR system	USA
16. Serological pipette 5, 25, 50 mL	USA
17. T75 Flasks	USA

18. Varioskan Flash Multimode ELISA reader	USA
19. Vortex Genie 2	USA
20. Water bath memmert	Germany
21. ZEISS LSM 800	Germany

APPENDIX B

CHEMICAL AND REAGENYS

1.	1,4-chrysene quinone	USA
2.	Absolute ethanol	USA
3.	Allophycocyanin (APC)-labeled CD86 antibody	USA
4.	Anti-CD11c antibody	USA
5.	Anti-F4/80 antibody	USA
6.	Bradford Bio-Rad Protein	USA
7.	Cryogel	USA
8.	DAPI (4′,6-diamidino-2-phenylindole)	USA
9.	Dulbecco's Modified Eagle Medium (DMEM)	USA
10.	Enzyme-linked immunosorbent assay (ELASA) kit	USA
11.	Fetal Bovine Serum (FBS)	USA
12.	Fluorescein isothiocyanate (FITC)-labeled CD206 antibody	USA
13.	Goat anti-mouse IgG`	USA
14.	High-Capacity cDNA Reverse Transcription Kit	USA
15.	High pure RNA isolation kit	Taiwan
16.	HEPES	USA
17.	Horse Serum	USA

18. Lipopolysaccharide (LPS; *Escherichia coli* 026: B6)	USA
19. Macrophage colony-stimulating factor (M-CSF)	USA
20. Multiscribe reverse transcriptase	USA
21. Normal saline	Thailand
22. Penicillin-Streptomycin	USA
23. Phosphate Buffer Saline (PBS)	Thailand
24. Primer	USA
25. PowerUp™ SYBR™ Green Master Mix	USA
26. RNase/DNase free H_2O	Germany
27. QuantiChrom Creatinine-Assay (DICT-500)	USA
28. Sodium pyruvate	USA
29. Tissue-Tek OCT compound	UK

APPENDIX C

REAGENTS PREPARATION

1. Blocking solution

5% FBS	500	µL
3% BSA	300	µL
0.01% Tween-20	1	µL
PBS	9.199	mL

2. 1,4-chrysene quinone (1,4-CQ)

Stock solution (1 mM)

1 4-chrysene quinone	2.5827	mg
ddH_20	10.0	mL

Stock solution (10 µM)

1 4-chrysene quinone (1mM)	10	µL
ddH_20	9.990	mL

Working solution (100 nM)

Stock solution (10 µM)	60	µL
cDMEM	5.940	mL

3. Coating solution

Na_2CO_3	0.356	g
$NaHCO_3$	0.84	g
ddH_20	100	mL

4. Complete DMEM (Dulbecco's Modified Eagle Medium (DMEM) with high glucose)

DMEM	43.350	mL
10%FBS	5	mL
1%Sodium pyruvate	500	µL
1%HERES	500	µL
1.3%Pen-strep	650	µL

5. Complete DMEM (Horse serum + m-csf)

DMEM	47.5	mL
Horse serum	2.5	mL
m-csf (conc. 1.250 µg)	12.5	µL

6. 1X DNase I Buffer

10 mM Tris-HCl	78.8	mg
2.5 mM $MgCl_2$	11.9	mg
0.5 mM $CaCl_2$	2.8	mg
ddH_20	50	mL

7. 70% ethanol

100% ethanol	70	mL
Sterile water	30	mL

8. FACS flow buffer

1X PBS	9.895	µL
1% FBS	100	µL
0.05% NaN3	5	µL

9. 1X Phosphate Buffer Saline (PBS)

Stock solution (10X PBS)	100	mL
ddH_20	900	mL

10. Lipopolysaccharide

Stock solution (10 µg)

Lipopolysaccharide (1 mg)	5	µL
ddH_20	495	µL

Working solution (100 ng)

Stock solution (10 µg)	60	µL
cDMEM	5.940	mL

11. Macrophage colony-stimulating factor (M-CSF)

Working solution (25 ng/mL)

Stock (50 µg)

ddH_20	500	µL

12. Primer

Working solution (10 mM)

Stock solution (100 mM)	20	µL
ddH_20	180	µL

13. Quantitative Real-Time Polymerase Chain Reaction (qPCR) master mix

10X RT Buffer	2.0	µL
25X dNTP Mix (100 mM)	0.8	µL
10X RT Random Primers	1.0	µL
MultiScribe™Reverse Transcriptase	1.0	µL
Nuclease-free H_2O	4.2	µL

14. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) master mix

PowerUp™SYBR™Green Master Mix (2X)	5.0	µL
Forward primer	0.2	µL
Reverse primer	0.2	µL
Nuclease-free H_2O	2.6	µL
cDNA	2.0	µL

15. Stop solution ELISA

2N H_2SO_4	2.805	mL
ddH_20	47.195	mL

16. 50X TAE buffer

Tris base	242	g
Glacial acetic acid	57.1	mL
0.5M EDTA, pH 8.0	100	mL
ddH_20 (Total volume)	1	L

17. Wash Buffer

1X PBS	1000	mL
0.05% Tween 20	500	µL

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