Comparative Response of Dendritic Cells to Pathogenic and Non-Pathogenic *Leptospira* spp.

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การเปรียบเทียบการตอบสนองของเซลล์เดนไดรติกต่อเชื้อเลปโตสไปราสายพันธุ์ก่อโรคและสายพันธุ์ไม่ก่อโรค

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บทคัดย่อ

โรคเลปโตสไปโรซิส หรือ โรคฉี่หนู เป็นโรคติดต่อจากสัตว์สู่คน เกิดจากคนสัมผัสเชื้อแบคเรียเลปโตสไปราจำแนกออกเป็น 3 กลุ่ม กลุ่มก่อโรค กลุ่มกึ่งก่อโรค และ กลุ่มไม่ก่อโรค สาเหตุของโรคเกิดจากคนสัมผัสเชื้อแบคเรียเลปโตสไปราสายพันธุ์ก่อโรคที่ปนเปื้อนในสิ่งแวดล้อม เชื้อเข้าสู่กระแสเลือดและกระจายไปสู่อวัยวะเป้าหมาย ปัจจุบันความรู้ความเข้าใจการตอบสนองภูมิคุ้มต่อเชื้อเลปโตสไปราทั้งสายพันธุ์ก่อโรคและไม่ก่อโรคมีขีดจำกัด เนื่องจาก ข้อจำกัดของสัตว์ทดลอง โดยเฉพาะน้ำยาและสารเคมีสำหรับศึกษาด้านภูมิคุ้มกันของแฮมเตอร์ และ สายพันธุ์หนูไมซ์สำหรับศึกษากลไกการเกิดโรค เซลล์เดนไดรติกเป็นเซลล์ภูมิคุ้มกันที่พบตามผิวหนัง จึงเป็นเซลล์แรกที่ตรวจพบเจอเชื้อเลปโตสไปรา หรือ เซลล์เดนไดรติกที่เจริญมาจากโมโนไซท์ในกระแสเลือดพบมากบริเวณเนื้อเยื่อที่อักเสบบริเวณหรือที่ติดเชื้อ โดยเซลล์ทั้งสองชนิดมีลักษณะและบทบาท คือจับกินเชื้อและนำชิ้นส่วนของเชื้อไปกระตุ้นเซลล์ภูมิคุ้มชนิดทีเพื่อทำหน้าที่กำจัดเชื้อ ดังนั้นเซลล์เดนไดรติกมีบทบาทที่สำคัญต่อเชื้อเลปโตสไปรา วัตถุประสงค์ของงานวิจัยนี้ เปรียบเทียบการตอบสนองของเซลล์เดนไดรติกระหว่างเชื้อเลปโตสไปราสายพันธุ์ก่อโรคและสายพันธุ์ไม่ก่อโรคในโฮสต์ที่มีความไวรับต่อโรคเลปโตสไปโรซิส จากการทดลอง หนูไมซ์สายพันธุ์ C3H/HeNJ อายุ 5 สัปดาห์ ติดเชื้อเลปโตสไปราสายพันธุ์ Pomona ปริมาณ $1X10^3$ และ $1X10^6$ เซลล์ พบว่า หนูไมซ์รอดชีวิตและไม่แสดงอาการของโรคเลปโตสไปโรซิส แต่อย่างไรก็ตาม หนูที่ติดเชื้อปริมาณ $1X10^6$ ตรวจพบเชื้อที่ไตและเกิดพยาธิสภาพที่ปอดและไต โดยเฉพาะไตมีพังผืดและขนาดโกลเมรูลัสเล็ก ดังนั้น หนูสายพันธุ์ C3H/HeNJ ติดเชื้อเลปโตสไปราปริมาณ $1X10^6$ เซลล์เหมาะนำมาศึกษาเกี่ยวอาการติดเชื้อแบบเรื้อรังของโรค เพื่อการศึกษาการตอบสนองของเซลล์เดนไดรติดต่อเชื้อเลปโตสไปราในโรคเลปโตสไปโรซิส งานวิจัยทำการศึกษาการตอบสนองของเซลล์เดนไดรติกที่เจริญมาจากโมโนไซท์ (MoDCs) จากตัวอย่างคนที่มีสุขภาพดี คนเป็นผู้รับเชื้อที่ไวต่อโรคเลปโตสไปโรซิส ผลการทดลองพบว่า เซลล์MoDCsจับกินเชื้อเลปโตสไปราทั้งสายพันธุ์ก่อโรคและไม่ก่อโรค อย่างไรก็ตาม เชื้อเลปโตสไปราสายพันธุ์ก่อโรคกระตุ้นการตายของMoDCs ลดการเจริญเติบโตตัวเต็มวัยของMoDCs ลดการแสดงออกของโมเลกุล CD80 และ CD83 บนผิวเซลล์ และสร้างไซโตคาย IL-6 ทำให้มีผลยับยั้งการแบ่งตัวของเซลล์ naïve $CD4^+$ T พร้อมทั้งกระตุ้นเซลล์ $CD4^+$ สร้างไซโตคายน์ IL-10 เหนี่ยวนำกลายเป็นเซลล์ชนิด regulatory T cells (Treg) ในทางตรงกันข้าม เชื้อเลปโตสไปราสายพันธุ์ไม่ก่อโรคกระตุ้นการเจริญเติบโตตัวเต็มวัยMoDCs และสร้างไซโตคายน์ชนิด IL-12p70 และ IL-10 มีผลกระตุ้นการแบ่งตัวของเซลล์ $CD4^+$ ให้สร้างไซโตคายน์ Interferon ชนิดแกมมาไปเหนี่ยวนำกลายเป็นเซลล์ T helper 1 (Th1) นอกจากนี้ผลการศึกษาระดับทรานสคริปโตม พบว่า เชื้อเลปโตสไปราสายพันธุ์ก่อโรคพบยีนที่เกี่ยวข้องกับการตายของเซลล์และเหนี่ยวนำของเซลล์Treg เชื้อเลปโตสไปราสายพันธุ์ไม่ก่อโรคพบยีนที่เกี่ยวข้องกับการกระตุ้นการทำงานของMoDCs และการกระตุ้นทำงานของเซลล์ Th1 ดังนั้น เชื้อเลปโตสไปราสายพันธุ์ก่อโรคลดประสิทธิภาพการทำงานของเซลล์ MoDCs และเหนียวนำให้เกิดเซลล์ Treg มีผลให้ความสามารถการกำจัดเชื้อลดลงและเชื้อสามารถแพร่กระจายไปยังอวัยวะเป้าหมายได้ ในขณะที่ สายพันธุ์ไม่ก่อโรคกระตุ้นประสิทธิภาพการทำงานของเซลล์ MoDCs เหนียวนำให้เกิดเซลล์ Th1 สำหรับเข้ามากำจัดเชื้อ

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Pratomporn Krangvichian : Comparative Response of Dendritic Cells to Pathogenic and Non-Pathogenic *Leptospira* spp.. Advisor: Assoc. Prof. KANITHA PATARAKUL, Ph.D. Co-advisor: Asst. Prof. PATCHAREE RITPRAJAK, Ph.D.,Prof. TANAPAT PALAGA, Ph.D.

Leptospirosis is a zoonotic disease caused by pathogenic *Leptospira* species, which consist of three groups and contain 4 subclades: pathogenic strains (P1), intermediate strains (P2), and non-pathogenic strains (S1 and S2). Infection of pathogenic leptospires occurs through contact with contaminated environments. Then, leptospires present in the bloodstream disseminate to target organs. Nowadays, the knowledge of the immune response to pathogenic and non-pathogenic leptospires during early infection is still limited. Due to the limitation of a susceptible host, immunological reagents for hamsters and strains of mice are limited and not available. Dendritic cells are the first cells to encounter leptospires found in the skin, while monocyte-derived dendritic cells (MoDCs) are found in the bloodstream in inflammatory areas. Therefore, the role of dendritic cells is important for leptospires infection. The major goal of this study is to compare the responses of dendritic cells to pathogenic and non-pathogenic leptospires in susceptible hosts. Five-week-old C3H/HeNJ mice were infected with either $1x10^3$ or $1x10^6$ inoculum dose of *Leptospira interrogans* serovar Pomona. All infected mice survived and did not develop an acute lethal infection. However, C3H/HeNJ mice infected with $1x10^6$ cells showed kidney colonization of leptospires and pathological changes in the lung and kidney, including kidney fibrosis and small glomerular size. Therefore, C3H/HeNJ mice may be used as an animal model for sublethal leptospirosis.

Human monocyte-derived dendritic cells were used as an in vitro model to study the response of dendritic cells to leptospires. Immature MoDCs phagocytosed a limited number of both pathogenic and non-pathogenic strains. The pathogenic strains significantly induced apoptotic cells, impaired maturation, and increased IL-10 production. MoDC impairment inhibited naive CD4 proliferation, produced IL-10, and induced regulatory T cells. In contrast, the non-pathogenic strains induced MoDC maturation, and increased IL-12p70 and IL-10 production, leading to CD4 proliferation, IFN- γ production, and Th1 cell induction. Moreover, the transcriptome analysis found that the pathogenic strains were associated with genes regulating apoptosis and regulatory T cells, while non-pathogenic strains were associated with genes regulating the maturation of MoDCs and Th1 cells. Therefore, the pathogenic strains might reduce the MoDC maturation and induce T reg, resulting in inefficient elimination of pathogenic leptospiral infection. In contrast, the non-pathogenic strains might increase MoDC maturation and Th1 response, leading to bacterial clearance

Field of Study:	Medical Microbiology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Pratomporn Krangvichian

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Relationship of Part I and Part II

The major objective of the thesis was to determine the role of dendritic cells in pathogenesis of leptospirosis in susceptible hosts by comparing the response of dendritic cells to pathogenic and non-pathogenic *Leptospira* spp.

Part I of the thesis aimed to compare the response of the human dendritic cells to pathogenic and non-pathogenic *Leptospira* spp. Humans are susceptible to natural infection of pathogenic *Leptospira*. Dendritic cells were generated *in vitro* from monocytes in human peripheral blood to become monocyte-derived dendritic cells (MoDCs). The difference in the response of MoDCs to pathogenic and non-pathogenic strains of *Leptospira* might reveal the functions of dendritic cells in pathogenesis of human leptospirosis.

Part II of the thesis aimed to evaluate the susceptibility of C3H/HeNJ mice to pathogenic *Leptospira* infection. In case of susceptibility, mouse dendritic cells might be further investigated for the response to *Leptospira* spp.

Two manuscripts are parts of the thesis to fulfill the completion of the degree of Doctor of Philosophy in Medical Microbiology.

Background and Rationales

Leptospirosis is a neglected tropical disease that has a global impact on public health and agricultural production (1). Recently, the genus *Leptospira* has been reclassified into four subclades: S1 and S2 (saprophytic), P2 (intermediate) and P1 (pathogenic) based on the average nucleotide identity of their genomes, which was used instead of the clusters classically named for *Leptospira* groups (2, 3). Saprophytic species can survive in environmental conditions such as soil and freshwater for several weeks. The new species of S2 subclade has been shown not to establish acute infection and renal colonization in hamsters. They generally cannot cause disease in both humans and animals. Intermediate species in P2 subclade have closer ancestry to pathogenic strains and typically cause mild infection in humans and animals. Pathogenic *Leptospira* in P1 subclade, of which dominant species is *L. interrogans*, may

cause severe leptospirosis in broad-spectrum animal and human hosts. Pathogenic *Leptospira* colonize the proximal tubules of the kidneys of reservoir hosts, especially rats and mice. Leptospires are then excreted into urine and contaminated the environment. After direct or indirect contact with infected urine, the spirochetes penetrate mucus membrane or skin abrasion, enter the bloodstream and disseminate to target organs leading to leptospirosis in susceptible hosts, mainly mammals. Humans serve as accidental hosts. The symptoms of leptospirosis in humans are a range of clinical presentations from mild disease such as flu-like illness to a more severe form such as icterus, pulmonary hemorrhage, kidney damage that may result in fatal outcome (1, 4, 5). The pathogenesis of leptospirosis has several questions that remain unanswered. Pathogenic leptospires not only evade but also stimulate the immune system as part of the host-pathogen interaction, which is essential for leptospirosis pathogenesis (6, 7). Most studies of innate immune response against leptospires focused on macrophages and neutrophils (7). Little is known about the response of dendritic cells to leptospires. Dendritic cells are professional antigen-presenting cells that are central to linking innate and adaptive immunity and play an important role in the induction of protective immune responses against pathogens (8-10). The main functions of dendritic cells are to phagocytose, process, and present antigens to adaptive immune cells followed by triggering their polarization into effector cells. Immature dendritic cells are found in the skin and mucosa, which are the first organs to interact with leptospires during disease transmission. Mature dendritic cells are essential in warning the host against entering pathogens by recognizing them through Pattern Recognition Receptors (PRRs), such as toll-like receptors, C-type lectins, and intracellular nucleic acid signals (11). Consequently, mature dendritic cells degrade the bacteria and present antigens to naïve T lymphocytes (9). So far, only one study reported that live virulent and attenuated strains of *L. interrogans* serovar Pyrogenes and Autumnalis induced maturation of monocyte-derived dendritic cells but had different effects on IL-12p70 and TNF-α production (12). The results revealed that two cytokines (IL-12p70 and TNF-α) are important for naive T cell development and adaptive immune response shaping (12). Several studies demonstrated that humoral

immune response is associated with protective immunity against leptospirosis (13). Moreover, previous studies reported that the non-pathogenic *L. biflexa* was involved in an early engagement of the innate immune system and induced a few chemoattractant (14). On the other hand, the pathogenic *L. interrogans* increased pro-inflammatory cytokines leading to the chemo-cytokine storm (14), commonly encountered in sepsis-like infection. Therefore, part I of the thesis aimed to compare the effect of pathogenic and non-pathogenic *Leptospira* spp. on human monocyte-derived dendritic cells. The study results might reveal the role of dendritic cells in leptospiral infection leading to a better understanding of immunopathogenesis and immune evasion of pathogenic *Leptospira* in humans.

Hamsters and guinea pigs were mostly used as susceptible models of leptospirosis. Previous studies demonstrated that hamsters and guinea pigs expressed various proinflammatory cytokines and chemokines in response to leptospiral infection (15-17). Due to the limitations of immunological reagents in hamsters and guinea pigs, knowledge of the immune response to *Leptospira* has been limited. Moreover, they are highly susceptible animals and rapidly die after infection, resulting in the difficulty of investigating immune response. Most mouse strains are resistant to pathogenic *Leptospira* infection and act as reservoir hosts (18, 19). Susceptibility to infection in humans may be associated with a lack of recognition of leptospiral lipopolysaccharide (LPS) by toll-like receptor 4 (TLR4) on human macrophages, whereas resistance in mice has been related to antibody production at 48-72 hours after infection (19) and the capacity of TLR4 on mouse macrophages to interact with leptospiral LPS (7, 20, 21). Previous studies demonstrated that C3H/HeJ mice (TLR4 defective mice) are considered slightly immunocompromised and are susceptible to lethal and sublethal leptospirosis (22-25). In addition, C57BL/6J TLR4 knockout mice, which cannot express TLR4, succumb to leptospiral infection (26). Nonetheless, a wide range of clinical outcomes depend on strain of *Leptospira*, age of tested mice, and inoculum dose (18). Accordingly, TLR4 on mouse macrophages plays an important role in controlling acute leptospirosis (27). Nevertheless, the comprehension of immune responses to pathogenic and non-pathogenic leptospires during early infection is still limited (16, 28). Chemokine attraction and cytokine production that recruited immune cells and

induced inflammation during early immune defense were different against pathogenic and non-pathogenic leptospires in C3H/HeJ mice (14). C3H/HeJ mice are not commercially available in Thailand and require expensive oversea purchasing and shipping. C3H/HeNJ mice, a cross breeding between C3H/HeJ (TLR4 defective mice) and C3H/HeN (wild-type TLR4) mice, are commercially available at a reasonable price but have not been tested for susceptibility to leptospirosis. Therefore, part II of the thesis aimed to evaluate the susceptibility of C3H/HeNJ mice infected with virulent *Leptospira interrogans* serovar Pomona.

Objectives

Part I:

Title of manuscript I: Comparative response of human monocyte- derived dendritic cells (MoDCs) to pathogenic and non-pathogenic *Leptospira* spp.

1. To compare the effect of pathogenic and non- pathogenic *Leptospira* spp, on maturation and cytokine production of MoDCs
2. To investigate the transcriptome profiling of MoDCs stimulated with pathogenic and nonpathogenic leptospires
3. To investigate the effect of pathogenic and non- pathogenic *Leptospira*-stimulated MoDCs on $CD4^+$ T cells

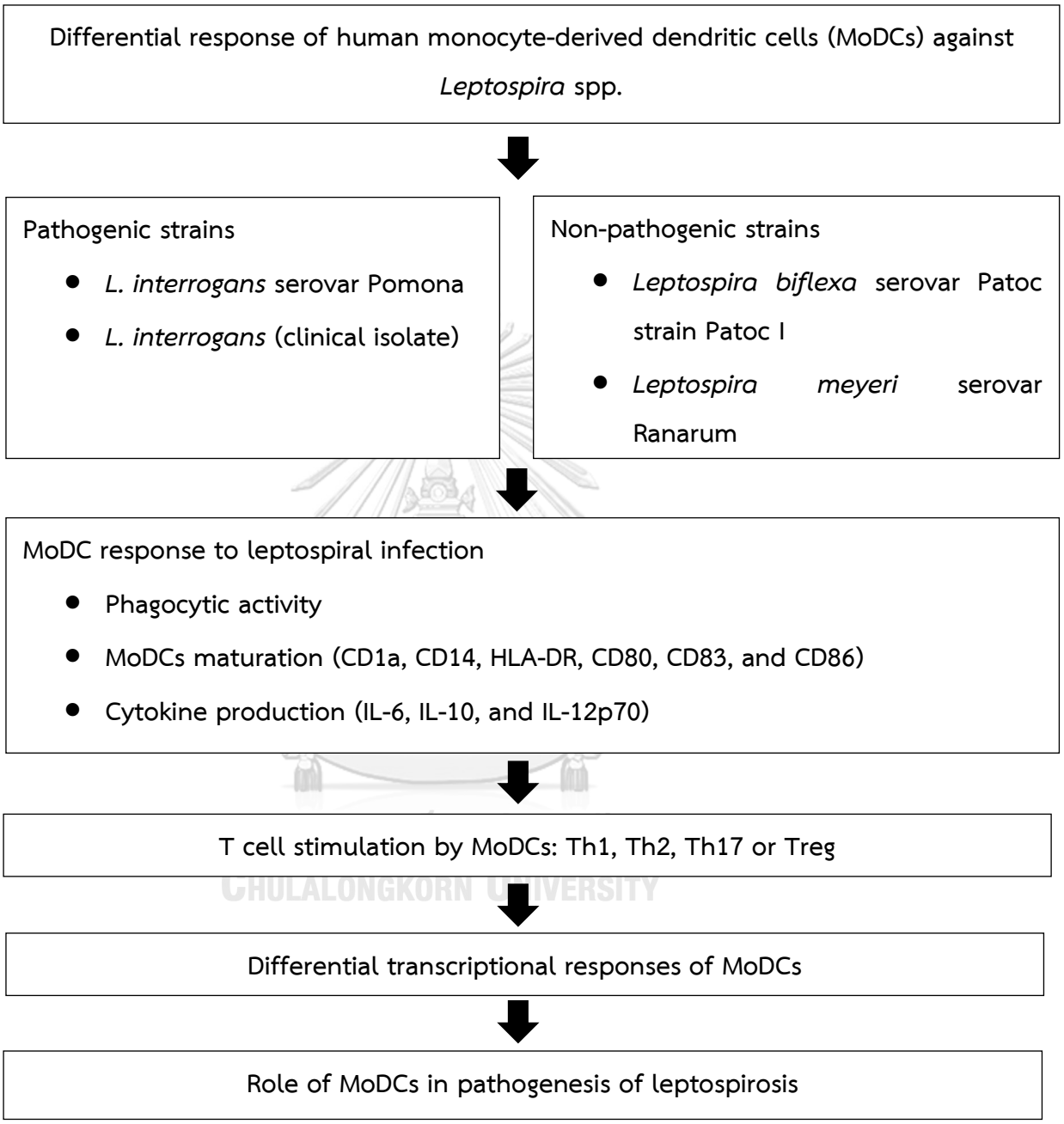
Part II:

Title of manuscript II: Sublethal infection of C3H/ HeNJ against *Leptospira interrogans* serovar Pomona

1. To evaluate the susceptibility of C3H/HeNJ to *L. interrogans* serovar Pomona

Conceptual framework

Part I:



Part II:

Female five-week-old C3H/HeNJ mice

↓

Intraperitoneal infection with 10^3 cells (low inoculum dose) and 10^6 cells (high inoculum dose) of *L. interrogans* serovar Pomona.

↓

Susceptibility and immune responses to *Leptospira* infection

- **Survival rate**
- **Leptospiral load in kidney**
- **Histopathology (kidney, lung, liver)**
- **Antibody levels**
- **Cytokine production (IFN-γ, IL-6 and IL-10)**

↓

Determine the susceptibility of C3H/HeNJ mice to leptospirosis

Benefits of the study

Part I: The comparison of the responses of human monocyte-derived dendritic cells between pathogenic and non- pathogenic *Leptospira* can provide a better understanding of the role of dendritic cells in leptospiral infection in humans. As an important link between innate and adaptive immunity, human dendritic cells may be targets for the development of new therapies and prevention in leptospirosis.

Part II: C3H/HeNJ mice were evaluated for susceptibility to leptospirosis. This animal model might be used to investigate the immunological mechanisms and comprehend the host immune responses against leptospires. In case of susceptibility, it can be useful to test the protective efficacy of new vaccines.

SECTION II

Part I: Comparative Response of Human Monocyte-derived Dendritic Cells to Pathogenic and Non-pathogenic *Leptospira* spp.

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Abstract

Leptospirosis has emerged as one of public health problems worldwide. The role of the host immune response in leptospirosis pathogenesis and prevention is obscure. Dendritic cells play an important cellular network that connect innate and adaptive immune response. Therefore, the aim of this study was to compare the function and phenotype of human monocyte derived dendritic cells (MoDCs) infected with live pathogenic (*Leptospira interrogans* serovar Pomona and a clinical isolate of *L. interrogans*) and non-pathogenic (*L. biflexa* and *L. meyeri* serovar Ranarum) leptospires. Even though the phagocytic activity of immature MoDCs against leptospiral strains varied, the pathogenic *Leptospira* significantly induced MoDC apoptosis higher than the non-pathogenic strains. However, MoDCs stimulated with the pathogenic leptospires expressed significantly lower level of CD80 and CD83 on the cell surface than those stimulated with the non-pathogenic leptospires. The pathogenic leptospires induced MoDCs to produce significantly higher IL-6 but lower IL-12p70 than the non-pathogenic leptospires. The pathogenic leptospires-stimulated MoDC significantly inhibited proliferation of autologous $CD4^+$ T cells and significantly induced IL-10 production in the $CD4^+$ T cells, which was associated with increased regulatory T cells. In contrast, the non-pathogenic-stimulated MoDCs significantly increased the level of IFN-γ that might lead to T-cells polarization toward predominant Th1-type response. In conclusion, the pathogenic Leptospira strains seemed to impair MoDC maturation and induce regulatory T cells possibly leading to immune evasion. In contrast, the non-pathogenic leptospires preferred activation of Th1 cells, which might be related to immune clearance. In this study, the difference in functional responses of human MoDCs against the pathogenic and the non-pathogenic leptospires might provide an important clue in the pathogenesis mechanism of leptospiral infection.

Keywords: monocyte-derived dendritic cells, immune response, *Leptospira*, leptospirosis

Introduction

Leptospirosis, caused by pathogenic Leptospira species, is a re-emerging zoonosis and one of public health concern (1, 29). *Leptospira* infection has a high incidence in tropical and subtropical regions (1). Human leptospirosis has been reported approximately 1.03 million cases per year (30) worldwide. *Leptospira* spp. are currently classified into 4 subclades based on phylogenomic analysis and the level of pathogenicity (2, 3). Pathogenic strains (P1 subclade) are commonly reported to cause leptospirosis in humans. Intermediate strains (P2 subclade) are unusual causes of leptospirosis and more closely related to the pathogenic strains. Non-pathogenic strains (S1 and S2 subclades) are saprophytic species, which naturally survive in the environment and are unable to cause leptospirosis. Pathogenic species colonize renal tubules of reservoir hosts including rodents and are excreted into urine leading to contaminated environments. Humans are susceptible to infection and become accidental hosts. Pathogenic *Leptospira* spp. can enter the human body through skin abrasion and mucous membrane resulting in various clinical manifestations from mild to severe and life-threatening infection, such as kidney and hepatic failure and pulmonary hemorrhagic syndrome, called Weil's disease (31).

The knowledge of host-pathogen interaction in leptospirosis remains poorly understood. Molecular Associated Molecular Patterns (MAMPs) of leptospires activate innate immune cells via Pattern Recognition Receptors (PRRs) resulting in the production of pro-inflammatory cytokines and chemokines that stimulate the immune system, and recruitment of immune cells (neutrophils, macrophage, and dendritic cells) to the infected sites (32). Neutrophils and macrophages can effectively phagocytose Leptospira by opsonization using specific antibodies against LPS and outer membrane proteins (33-35). However, pathogenic Leptospira are able to evade the host innate immune response. Previous studies found that lipopolysaccharide (LPS) of pathogenic Leptospira can escape activation through Toll-like receptor 4 (TLR4) of human macrophages and THP-1 cell line (20, 36). On the contrary, mouse macrophages recognized LPS via both TLR2 and TLR4 (21). Furthermore, C3H/HeJ mice (TLR4 defective) are susceptible to pathogenic Leptospira and represent a model of acute leptospirosis (22, 37, 38). Therefore, TLR4 plays an important role in the protection

and control of early *Leptospira* infection. As previously demonstrated, the primary protective immunity against *Leptospira* is the humoral immune response (19). In addition, previous studies demonstrated *L. interrogans* triggered a robust adaptive immune response leading to overwhelming increase of proinflammatory cytokines in infected C3H/HeJ mice (14). In terms of adaptive immunoprotection, heat-killed *L. borgpetersenii* vaccine induced a Th1 response and activated both CD4+ $\alpha\beta$ and WC1+ $\gamma\delta$ T proliferation (39, 40). Thus, it is likely that not only humoral-mediated immunity, but also cell-mediated immunity are important immune responses against Leptospira.

Dendritic cells (DCs) are professional antigen-presenting cells that link innate and adaptive immune systems. Immature DCs live in tissues throughout the body, such as skin and mucosa, where they recognize, capture, and process antigens. Then, immature DCs migrate to lymph nodes and become mature DCs. Mature DCs highly express co-stimulatory molecules and present the processed antigen to naïve T cells (8, 10, 41). Monocyte-derived dendritic cells (MoDCs) have usually been used for in vitro experiments. MoDCs show similarities in morphology, physiology, and function to conventional myeloid dendritic cells (42).

The role of DCs in leptospirosis is not well understood because of limited studies. Cell wall of *L. interrogans* possibly contained high mannose as shown by carbohydrate analysis using lectins, therefore *L. interrogans* might bind to dendritic cells via Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (12). Live virulent and attenuated strains of *L. interrogans* serovar Pyrogenes and Autumnalis induced MoDC maturation but had different effects on IL-10, IL- 12p70, and TNF-α production (12). Moreover, in C3H-HeJ mice *L. interrogans* serovar Copenhageni and *L. biflexa* serovar Patoc activated dendritic cells within 24 h after infection (14).

Humans are susceptible hosts of pathogenic *Leptospira* infections, resulting in acute and occasionally fatal infections (4). The knowledge of human dendritic cell response to leptospiral infection is needed to better understand its role in pathogenesis of leptospirosis. Therefore, this study aimed to compare the effect of pathogenic and non-pathogenic strains of *Leptospira* spp. on human MoDCs.

Materials and methods

Human blood samples

The buffy coats from 5 healthy blood donors were obtained from the Thai Red Cross Society (Bangkok, Thailand) after removal of plasma and platelets. The study was performed according to institutional guidelines and approved by the Institutional Ethics Committee (Approval Number: 12/2563).

Isolation of $CD14^+$ monocytes and $CD4^+$ T cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by density gradient centrifugation method using lymphocyte separation media (Biowest) (43). $CD14^+$ monocytes were separated from PBMC by positive selection using $CD14^-$ antibody coated magnetic beads according to the manufacturer's instruction (Miltenyi Biotec). $CD4^+$ T cells were separated from unlabeled CD14 monocytes by negative selection using Mojosort Human $CD4^+$ T cells isolation kit (BioLegend) following the manufacture's protocol. $CD4^+$ T cells were cryopreserved with RPMI 1640 containing 10% fetal bovine serum (FBS) (Gibco) and thawed before using in co-culture experiments.

Generation of monocyte-derived dendritic cells (MoDCs)

To generate immature MoDCs, $CD14^+$ monocytes (1×10^6 cell/ml) were cultured in 24 well flat-bottomed culture plates (Corning®) in a final volume of 1 ml of culture medium (RPMI 1640 with 10% heat inactivated FBS, 1% nonessential amino acids, 2mM L-glutamine, 1mM sodium pyruvate and 1% penicillin and streptomycin). Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) and recombinant human interleukin 4 (IL-4) (PeproTech) were added to the culture at a final concentration of 25 ng/ml, and half of the culture medium was replaced every 2 days with fresh culture medium containing GM-CSF and IL-4 at a final concentration of 25 ng/ ml (42). Plates of immature MoDCs were incubated at 37°C in humidified atmosphere containing 5% CO_2 for 7 days.

Leptospira and growth condition

Two pathogenic strains, *Leptospira interrogans* serovar Pomona and a clinical isolate of *L. interrrogans*, and two non-pathogenic strains, *L. biflexa* serovar Patoc strain Patoc I and *L. meyeri* serovar Ranarum, were used in this study. The clinical strain was isolated from blood of a leptospirosis patient at Surin hospital, Surin province, Thailand. Virulent strains (serovar Pomona and clinical strain) obtained by passage in golden Syrian hamsters (*Mesocricetus auratus*) followed by < 5 passages in vitro were used in the study. Leptospires were cultured in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (BD DifcoTM) supplemented with 10% bovine serum albumin at 30°C under aerobic condition until the exponential phase was reached at approximately 2 x 10^8 cells/ml (43). The bacterial cell number of leptospires were counted using a Petroff-Hausser chamber under a dark-field microscope.

Stimulation of monocyte-derived dendritic cells with _Leptospira_

Leptospires were washed three times with EMJH base medium and resuspended in RPMI 1640 medium at a final density of 2x10^8 cells/ml. On day 7 of MoDC culture, the bacterial suspension of 10^8 cells was added to 10^6 immature MoDCs at a multiplicity of infection (MOI) of 100 in 24-well flat bottom plates (Corning®) (12). Then, cell culture plates were centrifuged at 2500 xg for 15 min to synchronize the stimulation (44). MoDCs stimulated with 5 μg/ml of ultrapure *Escherichia coli* K12 lipopolysaccharide (LPS) (Invitrogen) were used for positive control and unstimulated MoDCs were used for negative control. After 24-h stimulation, culture supernatants were collected to measure cytokine production, and MoDCs were collected to determine cell viability, cell apoptosis, cell surface markers of maturation, and $CD4^+$ T cells differentiation.

MoDC viability was measured by dimethylathiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay using the CellTiter 96® Aqueous Non-Radiative Cell Proliferation assay (Promega) according to manufacture's instruction. The absorbance intensity measured by a microplate reader (Thermo Fisher Scientific) at 490 nm. The percentage of cell viability was calculated as follows: (Mean OD of sample – Mean OD of medium/ Mean OD of control – Mean OD of medium) x 100%.

To evaluate cell apoptosis, stimulated MoDCs were washed twice with cold phosphate buffered saline (PBS), resuspended in Annexin V Binding Buffer with FITC Annexin V and 7-Aminoactinomycin D (7-AAD), and then incubated for 15 min at room temperature (RT) in the dark according to the manufacture's instruction (BioLegend). In early apoptosis cells bound to both annexin V and 7-AAD, while those in late apoptosis cells bound to annexin V but not 7-AAD.

For surface marker analysis, stimulated MoDCs were counted, and plated at the number of 10^5 per well for each staining in 96 well plates (V-bottom) (Corning®). MoDCs were washed with PBS and incubated for 30 min with Zombie Red viability dye at RT (BioLegend). After that, cells were washed once with flow cytometry staining buffer (FACS) (1X PBS, 1% FBS and 0.1% Sodium azide) at 400 xg at 4°C for 10 min. Cells were stained with BV610-labeled anti-CD1a monoclonal antibody (mAb), APC-labeled anti-CD80 mAb, FITC-labeled anti-CD83 mAb, BV780-labeled anti-CD86 mAb, APC-A750-labeled anti-CD14 mAb and PC7-labeled anti-HLA-DR mAb at 4°C for 30 min. Finally, cells were washed with FACS buffer and fixed in 1% paraformaldehyde. All antibodies were purchased from BioLegend Company. Cells were assessed by flow cytometry. Graphs were plotted as relative geometric mean fluorescence intensity (MFI) calculated as the MFI of samples normalized by the MFI of the unstimulated MoDCs.

Co-culture of stimulated MoDCs and autologous $CD4^+$ T cells

After MoDCs stimulation for 24 h, $3x10^4$ cells of autologous $CD4^+$T cells were seeded in each well of a 96-well round bottom plate (Corning®), and cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin and streptomycin, and 40 ng/ml anti-CD3. $1.5x10^4$ cells of stimulated MoDCs ($CD4^+$T cells: stimulated MoDC at the ratio of 1:5) were then added to each well.

To determine intracellular cytokine production of $CD4^+$ T cells, stimulated MoDCs co-culture with autologous $CD4^+$ T cells in plates were incubated at 37°C for 24 and 48 h. During the final 5 h of culture, 5 ug/ml of brefeldin A (Sigma-Aldrich) was added to each well to prevent protein secretion from the Golgi apparatus. After that, cells were stained with Zombie Red viability dye and incubated for 30 min. Cells were

washed three times with FAC buffer and then were stained with BV610-labeled anti-CD1a mAb, anti-PE-labeled CD25 mAb and APC-A750-labeled anti-CD4 mAb. For IFN-γ, IL-4, IL-10, IL-17A staining, cells were fixed in 2% paraformaldehyde and permeabilized in 0.3% saponin. Then, cells were stained with PB450-labeled IFN-γ, APC-labeled IL-4, and PC7-labeled IL-17A. For IL-10 and Foxp3 staining, cells were fixed in Fix/Perm buffer and permeabilized with eBioscience buffer and stained with PE-labeled IL-10 and FIT-C-labeled FoXP3 according to the manufacturer's instruction. $CD4^+$ T cells were analyzed for intracellular cytokines by flow cytometry.

To investigate $CD4^+$ T cells proliferation induced by stimulated MoDCs, autologous $CD4^+$ T cells were stained with 5 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37°C for 20 min in the dark. After incubation, autologous $CD4^+$ T cells were washed three times by RPMI 1640 with 10% FBS to remove unbound CFSE. CFSE-labeled autologous $CD4^+$ T cells were co-cultured with stimulated MoDCs at the ratio of 1:5 for 4 days. The total number of $CD4^+$ T cells proliferation was analyzed by flow cytometry.

Phagocytic activity of MoDCs

Leptospira strains were stained with 10 μM of CFSE for 30 min at 37°C (45). Then, CFSE-stained leptospires were washed three times with PBS. Leptospires were counted and resuspended in RPMI 1640 at a density of 1×10^8 bacteria/ml. Before phagocytosis assay, CFSE-stained leptospires were aliquoted and then stained with 3.2 μM propidium iodide (PI). Leptospires incubated with PBS and heat-killed leptospires stained with PI were used as a negative control and a positive control, respectively. After that, CFSE-stained leptospires were co-cultured with stimulated MoDCs at MOI of 100. Plates were incubated at 37°C for 10 min and cells incubated at 4°C was used as a negative control. Phagocytic activity was stopped by adding ice-cold PBS. Then, cells were washed three times with FACS buffer and stained with PerCP5.5-labeled anti-CD1a mAb to analyze phagocytic activity by flow cytometry. The percentage of phagocytosis index was defined as the MFI multiplied by the fraction of $CFSE^+$ with $CD1a^+$ at 37°C divided by the fraction of $CFSE^+$ with $CD1a^+$ at 4°C (46). To confirm

phagocytosis by confocal microscopy, immature MoDCs were seeded on 12 nm round coverslips at a density of 10^6 cells/ml in 24-well plates (Corning®). The phagocytosis assay was performed as described above. Then, cells were fixed with 4% paraformaldehyde for 10 min at RT and then washed 3 times with PBS. Next, cells were permeabilized by 1% Triton X-100 in PBS for 10 min. Cells were then stained with Evans blue dye (Sigma) to stain the cell cytoplasm and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) to stain the nucleus. Coverslips were mounted with slow-fade diamond antifade on slide (Invitrogen) before imaging. All images were performed under a Zeiss LSM 800 confocal microscope using a 100x and 200x magnification.

Flow cytometry analysis

After staining as described above for each experiment, flow cytometry was performed using a CytoFLEX; 3 lasers and 13 colors Facscalibur (BD biosciences). The data was processed using Flow-Jo software (Version 10). Whenever relevant, isotype-matched control samples or fluorescence minus one sample was included in staining procedure.

Cytokine production of stimulated MoDCs

Culture supernatants of stimulated MoDCs were stored at -80°C. All samples were thawed and run in the same batch to quantify cytokine concentration. IL-6, IL-10 and IL-12p70 cytokines in the supernatants were measured using commercial ELISA kit (BioLegend) following the manufacture's instruction. Briefly, 96-well plates were coated with capture antibody solution and incubated overnight at 4°C. Plates were washed 4 times with washing buffer (PBS with 0.05%Tween-20) after each subsequent step. Assay diluent (10% BSA in PBS) was added in each well and the plates were incubated at 37°C for 1 h. Subsequently, standard dilution and samples diluted with assay diluent were added into the wells and incubated at 37°C for 2 h. Detection antibody solution was added and incubated at 37°C for 1 h. Avidin-horseradish peroxidase solution was added and incubated at 37°C for 30 min. Color was developed using 3,3'5,5'-tetramethylbenzidine substrate solution and the reaction was stopped

by using 2N sulfuric acid. The absorbance intensity was measured by a microplate reader at 450 nm. The cytokine concentration in the samples was calculated with standard curve and multiplied by dilution factor.

RNA extraction and quantification

Total RNA (n=3/experimental group) was extracted from stimulated MoDC at 4 h post-stimulation with TRIZOL® reagent (Life Technologies Inc.) and then purified using with RNeasy columns (Qiagen) according to the manufacturer's instruction. RNA samples were quantitated using a NanoDrop (ND-2000 spectrophotometer) and QubitTM RNA high sensitivity (Thermo Scientific).

Data Processing and transcriptome analysis of stimulated MoDCs

Experimental samples were analyzed using RNA sequencing (RNA-Seq, BGI Genomics) and then expressed genes were annotated by bioinformatics analysis to compare differentially expressed mRNAs of stimulated MoDCs and unstimulated MoDCs. After that, the differentially expressed genes (DEGs) of pathogenic and non-pathogenic strains were compared. The resulting p-values < 0.05 were adjusted using the Benjamini and Hochberg approach to control the false discovery rate (FDR). The DEGs of metabolic pathways were analyzed using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (47).

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Statistical analysis

Statistical significance was analyzed using the GraphPad Prism software (version 8, Chulalongkorn University license). The differences in parameters between groups determined using the two-way analysis of variance (ANOVA). P-values < 0.05 were considered statistically significant.

Results

The phagocytic activity of immature MoDCs against leptospires

Immature MoDCs are known to have higher phagocytic capacity than mature MoDCs (8). To evaluate phagocytic activity of immature MoDCs against different strains of leptospires, freshly isolated immature MoDCs were incubated with each strain of leptospires for 10 min. The effect of CFSE on viability of leptospires was previously determined. More than 95% CFSE-labeled leptospires were viable as shown by flow cytometry and fluorescence microscopy (S1 Fig). Fluorescence signals were measured to determine the uptake of CFSE-labeled leptospires into MoDCs (S2 Fig). The phagocytic index of immature MoDCs to ingest CFSE-labeled leptospires was dose-dependent (increased at higher MOI) (Fig 1). At an MOI of 100, the phagocytic index of the serovar Ranarum group was the highest among that of the pathogenic (serovar Pomona and clinical isolate) and the serovar Patoc groups, suggesting that the phagocytic activity of immature MoDCs against leptospires was strain dependent.

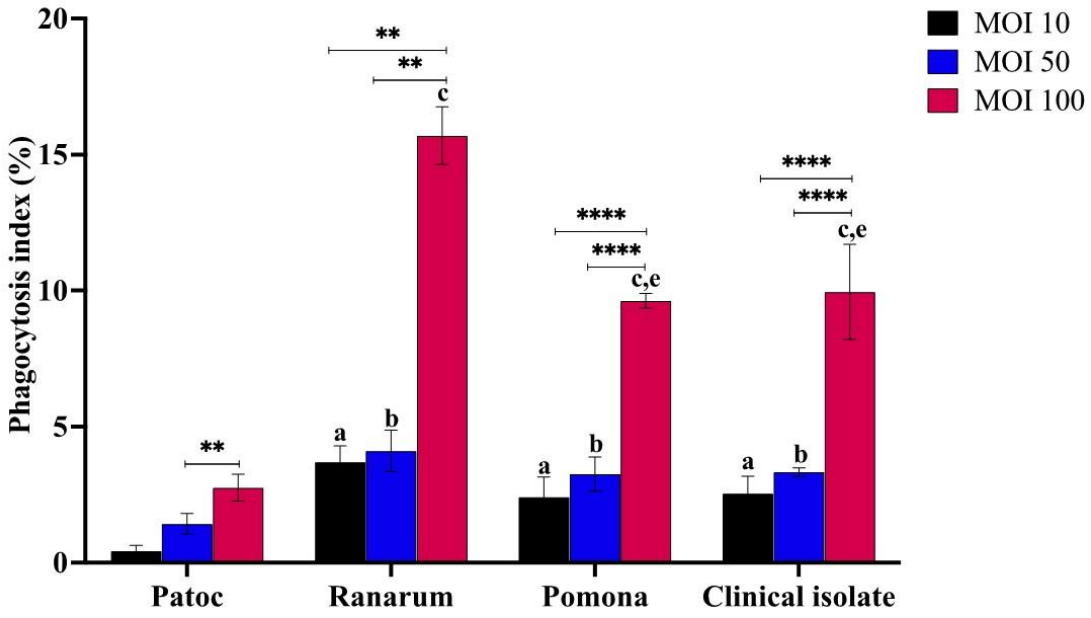


Figure 1 The phagocytic index of immature MoDCs against the non-pathogenic strains (serovar Patoc and Ranarum) and the pathogenic strains (serovar Pomona and clinical isolate) of leptospires.

Immature MoDCs were incubated with CFSE-labeled leptospires at various MOI (10, 50, and 100) and evaluated for phagocytic activity by flow cytometry. After incubation at 37 °C for 10 min, the interaction of MoDCs with CFSE-labeled leptospires was stopped

by cold PBS. MoDCs infected with CFSE- labeled leptospires immediately determined fluorescence signals (CD1a+ and CFSE+) by flow cytometry. The percentage of phagocytosis index was calculated as [(% positive cell x MFI at 37 °C divided by % positive cell x MFI at 4°C) x 100]. Each bar represents the mean ± SD from three donors. Statistical values were analyzed by a two-way ANOVA test. Asterisks denote significant differences between the MOI of each group. ** $P < 0.01$ and **** $P < 0.0001$. Letters a, b, and c indicate significant differences compared to the serovar Patoc at MOI of 10, 50, and 100, respectively. Letter e indicates significant differences compared to the serovar Ranarum at an MOI of 100.

Furthermore, immature MoDCs were examined for phagocytosis of CFSE-labeled leptospires by confocal microscopy (Fig 2). After 10-min incubation at an MOI of 100, a small number of CFSE-labeled leptospires were observed in the cytoplasm of MoDCs. Therefore, immature MoDCs displayed modest phagocytic activity against pathogenic and non-pathogenic strains. Interestingly, the pathogenic strains-activated MoDCs rapidly presented long dendrites and cytoplasmic processes, the characteristic of mature MoDC. In contrast, MoDCs stimulated with non-pathogenic strains remained in the round and symmetrical shape of immature MoDCs (Fig 2).

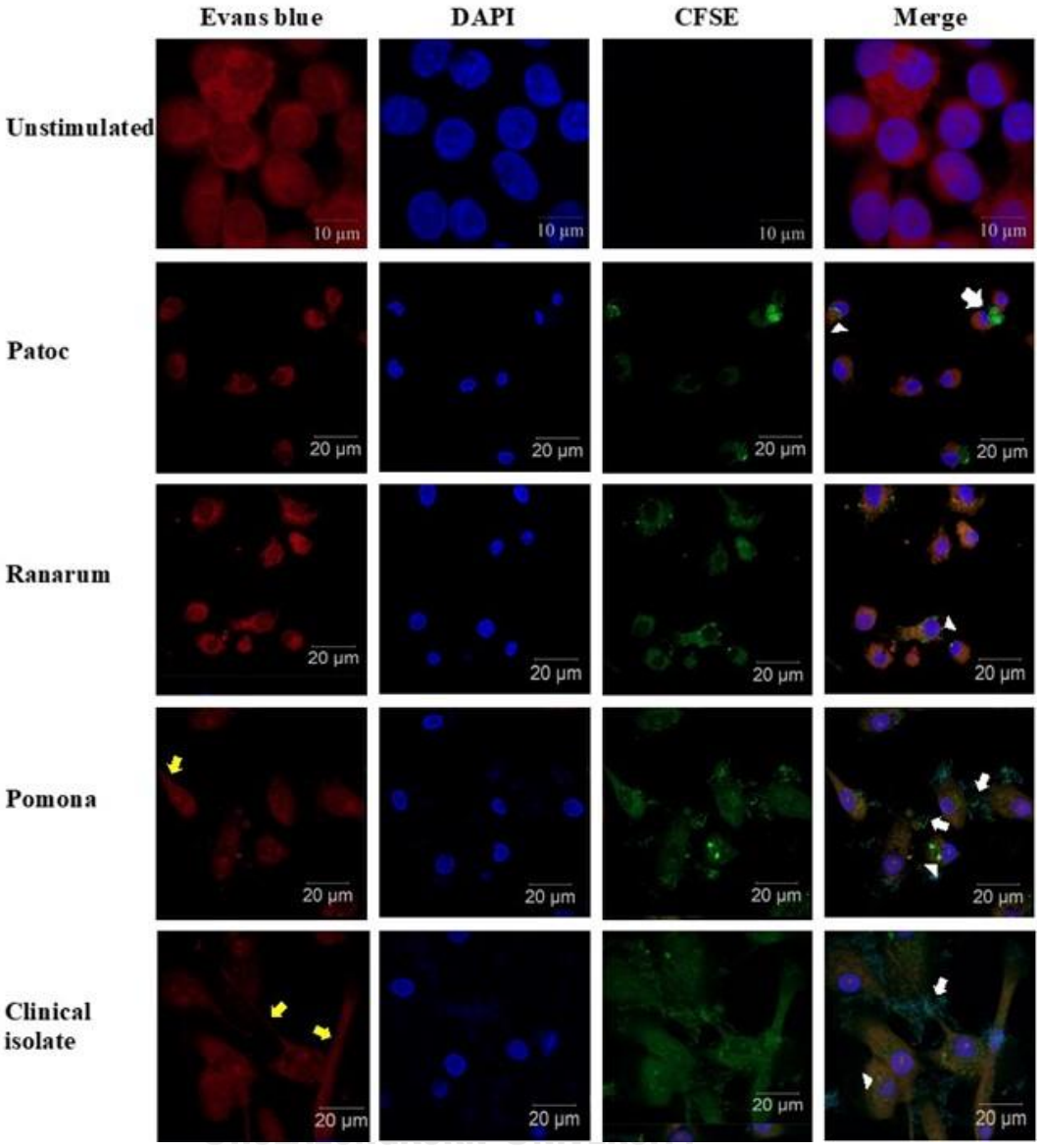


Figure 2 *Confocal microscopy of immature MoDCs infected with non-pathogenic strains (serovar Patoc and Ranarum) and pathogenic strains (serovar Pomona and clinical isolate) of leptospires at an MOI of 100 for 10 min compared with the unstimulated group (negative control).*

The cytoplasm was stained with Evans blue (red), the nucleus was stained with DAPI (blue) and leptospires were stained with CFSE (green). The all leptospires located inside the cells are indicated by white head arrows. The pathogenic strains outside the cells are indicated by white arrows. Long dendrites and cytoplasm processes of MoDCs are

indicated by yellow arrows. The scale bar of the unstimulated and all leptospires groups is 10 μM and 20 μM, respectively. Images are representatives of three donors.

MoDC apoptosis induced by leptospires

The cytotoxic effect of leptospiral infection on MoDCs at 24 h was determined by the MTS assay. At an MOI of 100, the pathogenic strains induced significantly higher level of MoDC death compared with the non-pathogenic strains (Fig 3A). Then, we determined whether cell death was due to apoptosis by annexin V and 7-ADD staining and flow cytometry analysis (Fig 3B). MoDCs infected with both strains of pathogenic leptospires presented significantly greater level of early apoptosis profile (Annexin V^+ 7-ADD^-) in comparison with those infected with the non-pathogenic strains (Fig 11C). The average level of apoptosis caused by the pathogenic and the non-pathogenic strains was approximately 10% and 3%, respectively. The percentage of apoptotic cells of uninfected MoDCs was 1%. Therefore, the pathogenic strains induced apoptosis of MoDCs more than the non-pathogenic strains.

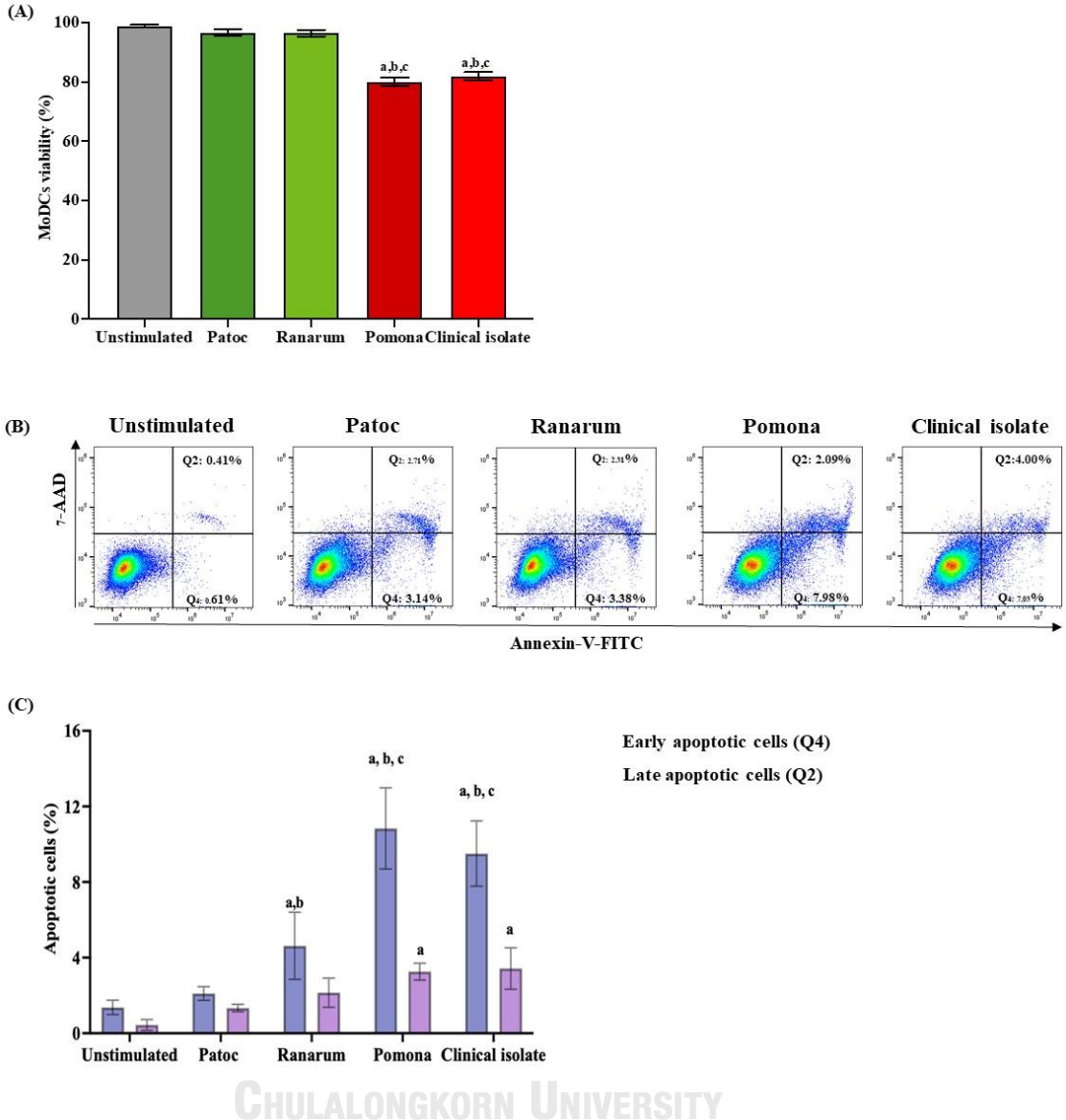


Figure 3 *The effect of the non-pathogenic strains (serovar Patoc and Ranarum) and the pathogenic strains (serovar Pomona and clinical isolate) on the viability of MoDCs.*

The unstimulated group was used as a negative control. MoDCs were stimulated with leptospires at an MOI of 100 for 24 h. (A) The percentage of viable MoDCs stimulated with leptospires using the MTS assay. The viability was compared with unstimulated MoDCs. (B) MoDCs apoptosis induced by leptospires. Scatter plots represents the stage of early apoptosis cells (Q4; Annexin V^+ 7-ADD^-) and late apoptosis cells (Q2; Annexin V^+ 7-ADD^+) of five donors. (C) The percentage of apoptotic MoDCs induced by leptospires. Each bar represents the mean ± SD of five donors. Statistical values were

analyzed by a two-way ANOVA test. Letters a, b, and c indicate significant differences compared with the unstimulated MoDCs, serovar Patoc, and serovar Ranarum, respectively.

MoDC maturation stimulated with leptospires

Monocytes were isolated from peripheral blood mononuclear cells, generated into immature MoDCs, and then stimulated by leptospires to differentiate to mature MoDCs. The effect of the pathogenic leptospires on MoDC maturation was compared with that of the non-pathogenic strains at an MOI of 100 for 24 h. The purity of cells was determined by detection of specific surface markers using flow cytometry. The purity of monocytes ($CD14^+$) and immature MoDCs ($CD1a^+$) was greater than 90% after generation (S3 Fig). In flow cytometry assay, $CD1a^+$ was used to gate population of MoDCs for the cell surface expression (Fig 4A). Mature MoDCs were identified by the loss of CD14 and increased expression of CD83 and co-stimulatory molecules (CD80, CD86, and HLA-DR). MoDCs stimulated by each leptospiral strain expressed significantly higher level of CD80, CD83, and CD86 than unstimulated MoDCs. Neither all leptospires nor the LPS of *E. coli* had any effect on CD1a or CD14 expression (Fig 4B and 4C). Interestingly, CD80 and CD83 on MoDCs activated by the pathogenic strains were significantly lower compared with those activated by the non-pathogenic strains. Additionally, no significant differences in CD86 and HLA-DR expression between MoDCs stimulated by the non-pathogenic and the pathogenic leptospires (Fig 4B and 4C). Thus, both non-pathogenic and pathogenic strains were able to induce MoDC maturation but resulted in different levels of CD80 and CD83 on the mature MoDCs.

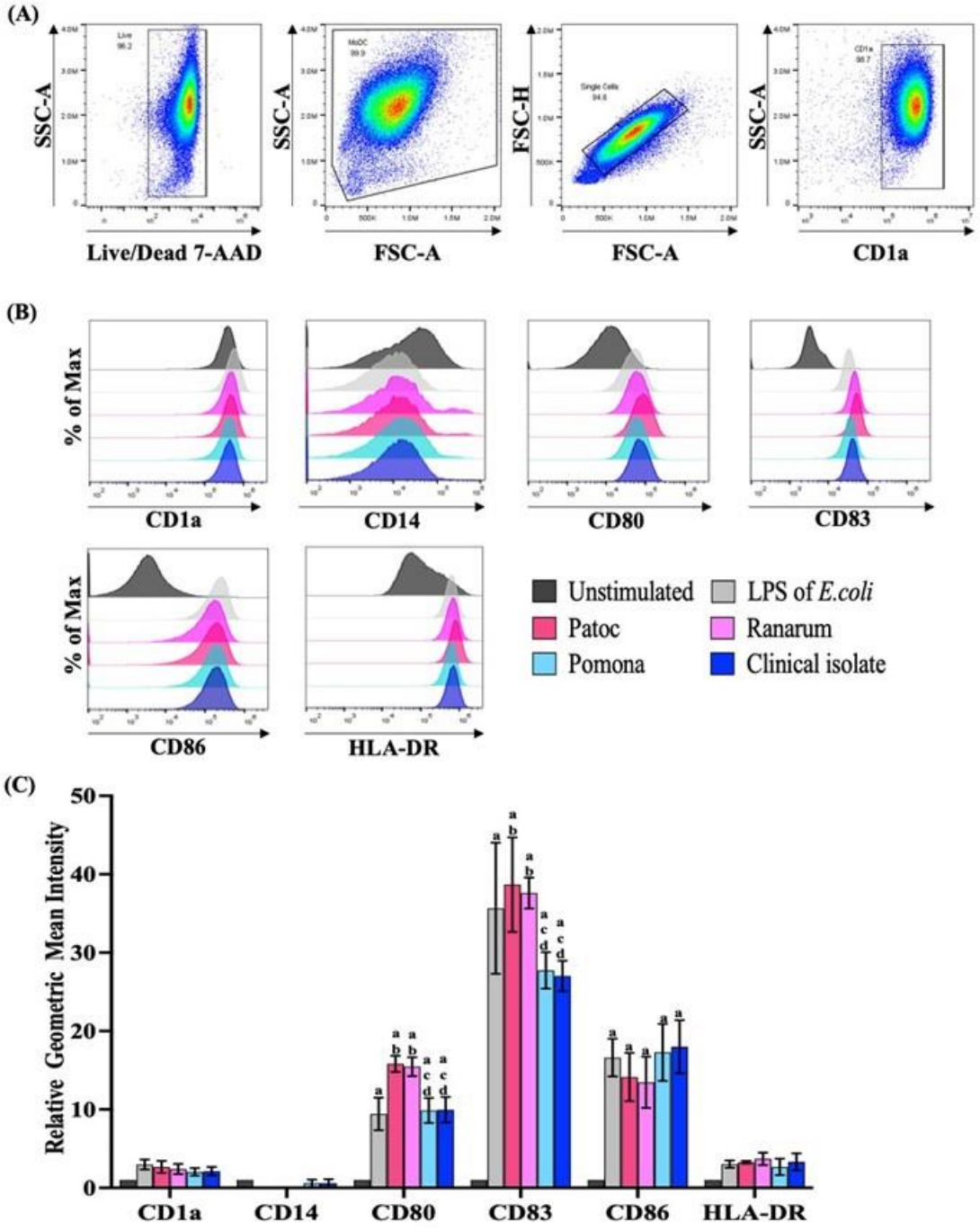


Figure 4 *MoDC maturation induced by the non-pathogenic strains (serovar Patoc and Ranarum), the pathogenic strains (serovar Pomona and clinical isolate), and LPS from E. coli.*

Uninfected MoDCs were used as a negative control. Cell surface markers were measured by flow cytometry after 24-h stimulation. (A) The expression of CD1a was gated according to Live/ Dead staining along with FSC and SSC parameters. (B)

Histograms depict the level of surface expression from a representative experiment. (C) The relative mean fluorescence intensity of surface markers on MoDCs from five donors. Each bar represents the mean ± SD from five donors. Statistical values were analyzed by a two-way ANOVA test. Letters a, b, c, and d indicate significant differences compared with the unstimulated MoDCs, LPS from E. coil, serovar Patoc, and serovar Ranarum, respectively.

Cytokine production of MoDCs induced by leptospires

IL-6, IL-12, and IL-10 production was evaluated in the culture supernatants obtained from MoDCs stimulated for 24 h with the non-pathogenic strains, the pathogenic strains, and *E. coli* LPS. Unstimulated MoDCs were used as a negative control. The pathogenic strains stimulated MoDCs to produce significantly lower amount of IL-12 and IL-10 but higher level of IL-6 than the non-pathogenic strains (Fig 5).

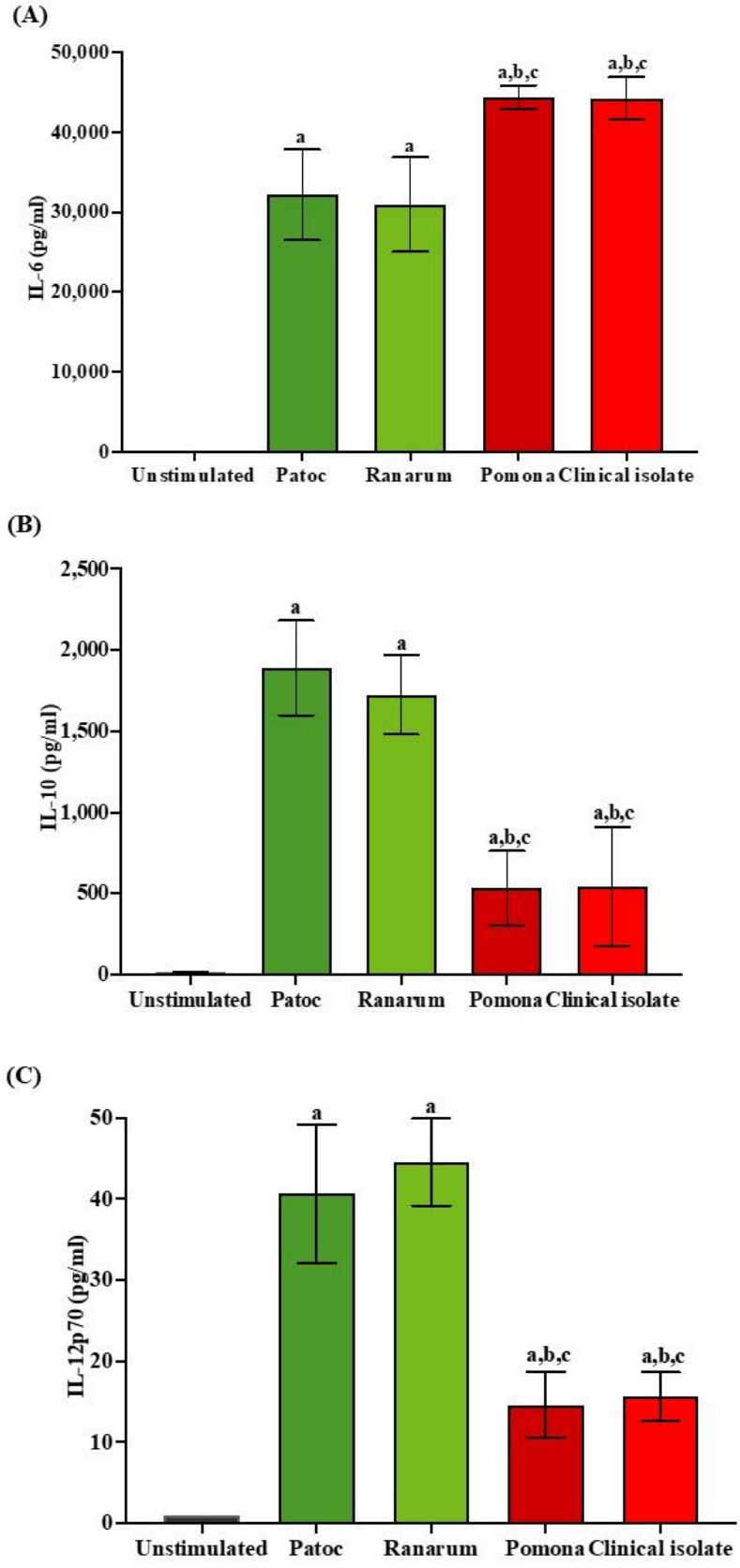


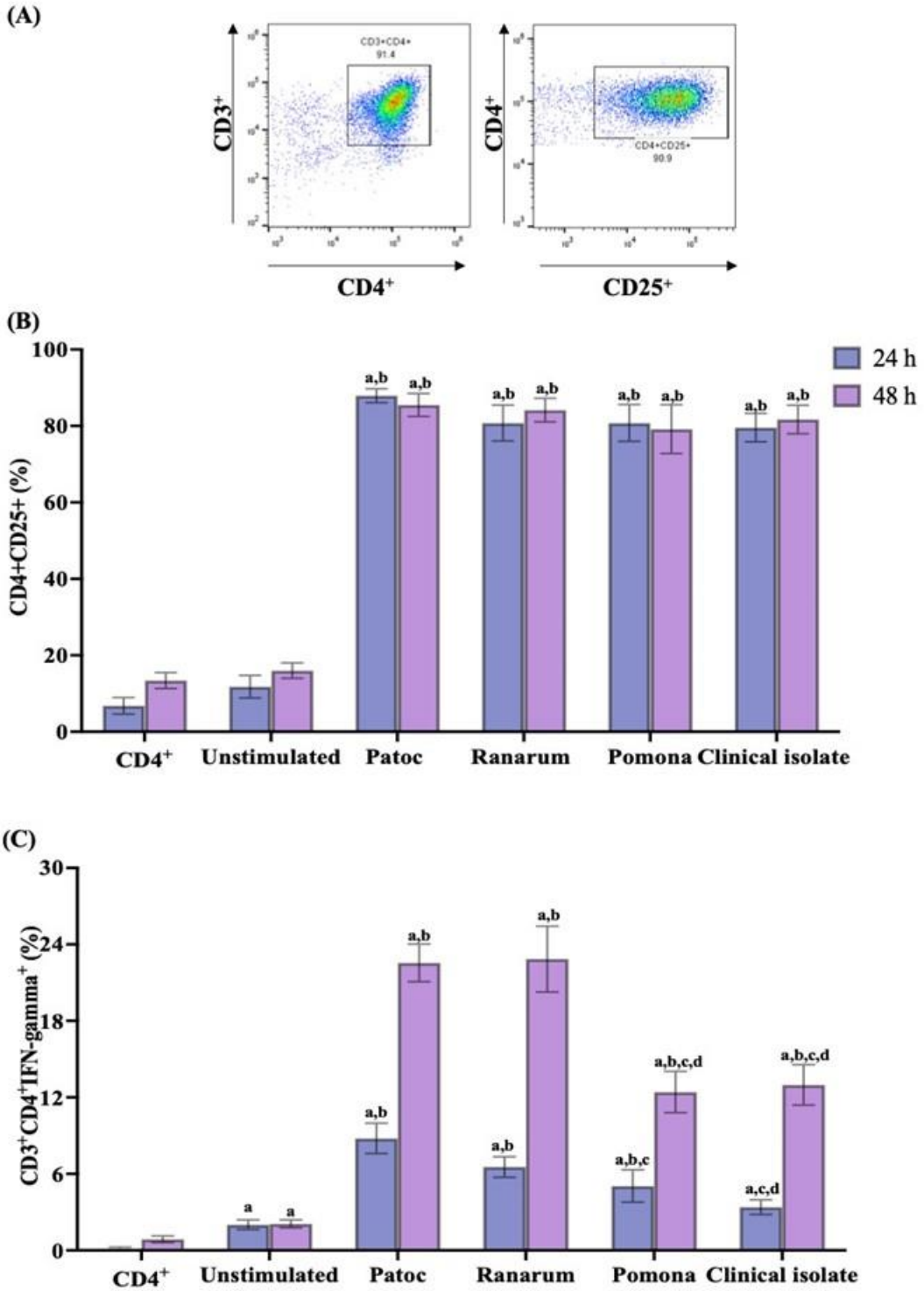
Figure 5 *Cytokine production of MoDCs induced by the non-pathogenic strains*

(serovar Patoc and serovar Ranarum) and the pathogenic strains (serovar Pomona and clinical isolate) at an MOI of 100 for 24 h.

IL-6 (A), IL-10 (B) and IL-12p70 (C) in the culture supernatants were measured by ELISA. Unstimulated MoDCs were used as a negative control. Each bar represents the mean ± SD of five donors. Statistical values were analyzed by a two-way ANOVA test. Letters a, b, and c indicate significant differences compared with the unstimulated MoDCs, serovar Patoc and serovar Ranarum, respectively.

Autologous T cell activation and proliferation by *Leptospira*-stimulated MoDCs

Autologous $CD4^+$ T cells isolation were stained with anti-CD4 mAb to evaluate the cell purity by flow cytometry. The purity of autologous $CD4^+$ T cells was found to be more than 85% (S4 Fig). To assess the T-cell stimulatory potential of the MoDC maturation, allogenic naïve T cells were utilized as responders. When leptospires-stimulated MoDCs were used as stimulators, a population of $CD4^+$T cells became activated, as determined by the expression of CD25 (Fig 6A and 6B). Next, the levels of IFN-γ-, IL-4-, IL-10-, and IL-17-expressing $CD4^+$ T cells were measured by intracellular cytokine staining assay. After 24-h and 48-h co-culture, the non-pathogenic strains-stimulated MoDCs were able to induce higher IFN-γ-producing $CD4^+$ T cells than the pathogenic strains-stimulated MoDCs (Fig 6C). This suggests that autologous $CD4^+$ T cells activated by the non-pathogenic strains-stimulated MoDCs have a predominant Th1 phenotype. In contrast, $CD4^+$ T cells secreted significantly higher level of IL-10 after 48-h stimulation with the pathogenic strains (Fig 6D). IL-4 and IL-17A were almost undetectable in all groups. Based on low expression of CD83 on MoDCs stimulated with the pathogenic strains, the population of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) was subsequently investigated. We found that the pathogenic strains-stimulated MoDCs significantly induced a higher number of Tregs than the non-pathogenic strains at 48-h stimulation (Fig 6E).


(A)
CD3+CD4+
91.4
CD4+CD25+
90.9
CD3+
CD4+
CD4+
CD25+
(B)
24 h
48 h
CD4+CD25+ (%)
100
80
60
40
20
0
a,b
CD4+
Unstimulated
Patoc
Ranarum
Pomona
Clinical isolate
(C)
CD3+CD4+IFN-gamma+ (%)
30
24
18
12
6
0
a
a,b
a,b,c
a,b,c,d
a,c,d
CD4+
Unstimulated
Patoc
Ranarum
Pomona
Clinical isolate

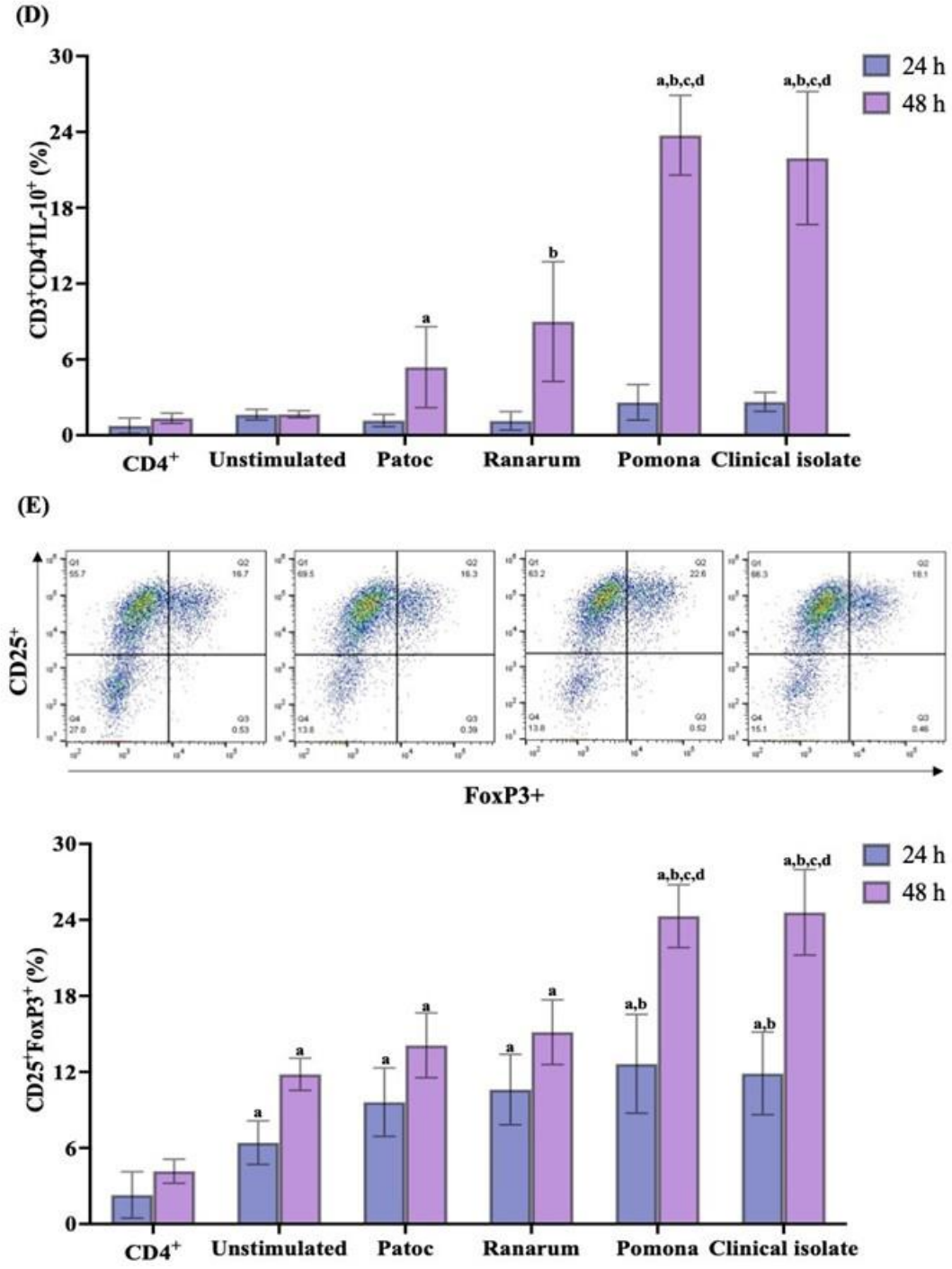


Figure 6 *The effect of MoDCs stimulated with the non-pathogenic strains (serovar Patoc and Ranarum) and the pathogenic strains (serovar Pomona and clinical isolate) on cytokine production of autologous CD4⁺ T cells at 24 and 48 h.*

(A) Representative flow cytometry dot plot showing the percentage of $CD4^+$ T cell activation of each group to determine the intracellular cytokines. (B) The percentage

of $CD4^+$ $CD25^+$ activated T cells. Intracellular staining of (C) IFN-γ and (D) IL-10 produced in autologous $CD4^+$ T cells (E) Dot plot and the percentage of $CD25^+FoxP3^+$ regulatory T cells at 48 h. Each bar represents the mean ± SD of five donors. Statistical values were analyzed by a two-way ANOVA test. Letters a, b, c, and d indicate significant differences compared with the $CD4^+$ T cells, unstimulated MoDCs, serovar Patoc and serovar Ranarum, respectively.

To evaluate the effect of leptospires-stimulated MoDCs on autologous $CD4^+$ T cell proliferation, leptospires-stimulated MoDCs were mixed with autologous $CD4^+$ T cells and $CD4^+$ T cell proliferation was measured by flow cytometry (Fig 7A-F). The MoDCs infected with the non-pathogenic strains induced significantly higher $CD4^+$ T cell proliferation than those infected with the pathogenic strains. Interestingly, the pathogenic strains-stimulated MoDCs slightly induced $CD4^+$ T cell proliferation, similar to unstimulated MoDCs. (Fig. 7G). Therefore, the pathogenic strains might induce high levels of IL-10 and Tregs, leading to inhibition of $CD4^+$ T cell proliferation. In contrast, the non-pathogenic strains might have better capacity to induce the proliferation of IFN-γ-producing $CD4^+$ T cells, Th1-type response.

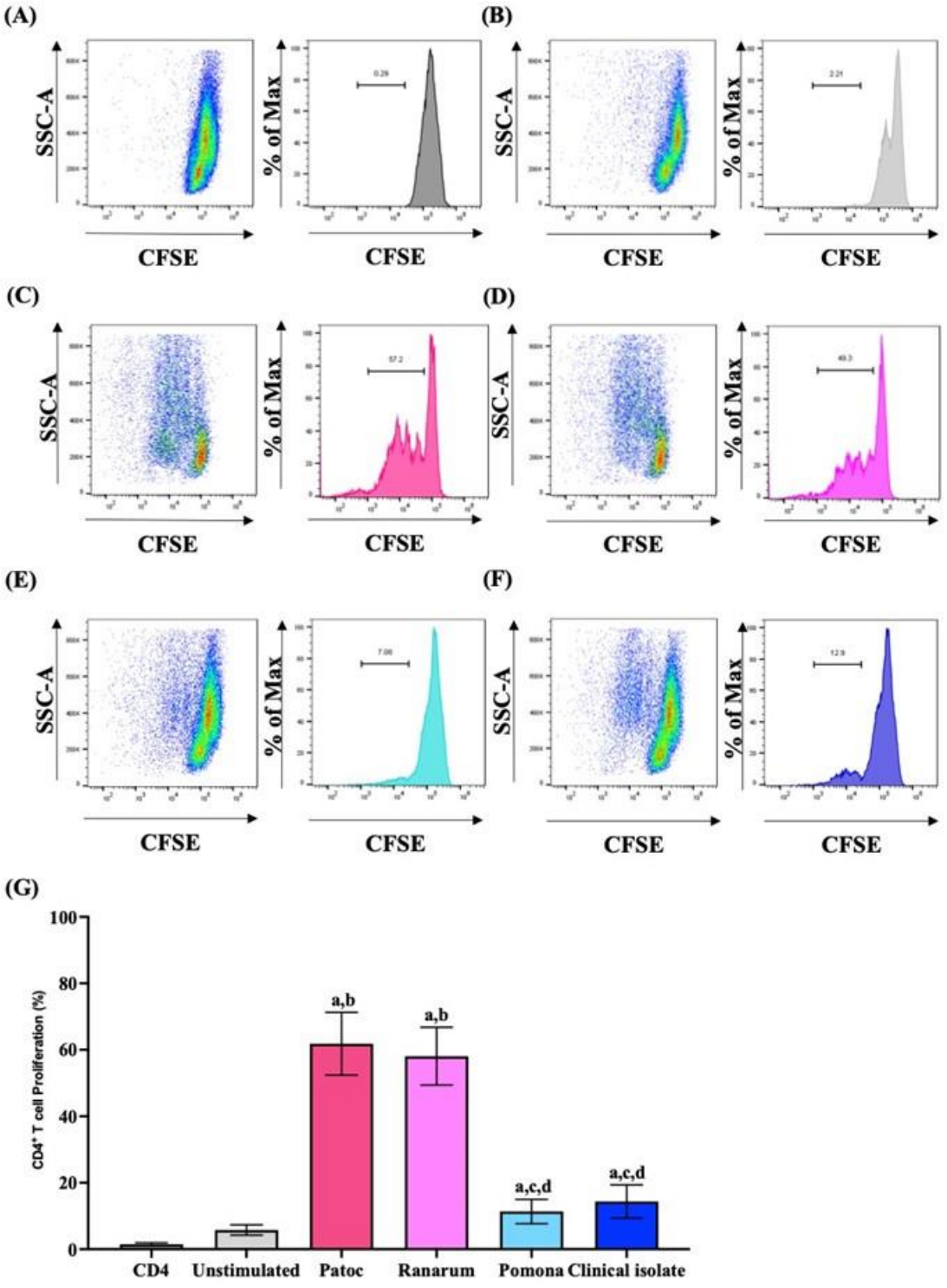


Figure 7 *Induction of autologous CD4$^+$ T cell proliferation by MoDCs stimulated with the non-pathogenic strains (serovar Patoc and Ranarum) and the pathogenic strains (serovar Pomona and clinical isolate).*

Stimulated MoDCs were co-cultured with autologous $CD4^+$ T cells at a ratio of 1:5 for 4 days and $CD4^+$ T cell proliferation was analyzed by flow cytometry. (A-F) The dot plot and histograms of $CD4^+$ proliferation in autologous $CD4^+$ T cells (A), and unstimulated MoDCs (B), serovar Patoc (C), Ranarum (D), Pomona (E), and clinical isolate (F) were determined fluorescence CFSE of $CD4^+$ T cells. (G) The percentage of autologous $CD4^+$ T cell proliferation induced by MoDCs stimulated with different leptospiral strains. Each bar represents the mean ± SD of five donors. Statistical values were analyzed by a two-way ANOVA test. Letters a, b, c, and d indicate significant differences compared with the autologous $CD4^+$ T cells, unstimulated MoDCs, serovar Patoc and serovar Ranarum, respectively.

Transcriptome profiles of MoDCs stimulated with leptospires

The response of MoDCs stimulated with the non- pathogenic and the pathogenic strains for 4 h was investigated by transcriptome analysis. The purified RNA obtained from all samples of stimulated MoDCs showed RNA integrity number above 8 (S5 Fig). The RT-qPCR was performed to determine the cytokine gene expression to select the optimal time point of MoDCs stimulated with leptospires for RNA sequencing. The results demonstrated that the non-pathogenic strains had a higher level of IL-1β gene expression than the pathogenic strainsat 4 h (S6 Fig), therefore RNA of MoDCs stimulated with leptospires for 4 h was used for transcriptomics analysis. The expression data of three donors in each group were analyzed to determine the average gene expression. Differentially expressed genes (DEGs) was identified for each sample group; MoDCs stimulated with serovar Patoc, serovar Ranarum, serovar Pomona, and clinical isolate, by comparison with the expression levels of uninfected samples (FDR-adjusted $p<0.05$, fold change ± 1.0). At FDR-adjusted p 0.05, only a small number of DEGs in both groups were retrieved, which was difficult to acquire a meaningful interpretation. Therefore, we adjusted FDR to $p<0.3$, which made the result consistent with previous RT-qPCR in term of IL-1β gene inclusion.

The non-pathogenic serovar Patoc modulated 501 genes (28 up-regulated and 473 down-regulated), the non-pathogenic serovar Ranarum modulated 26 genes (13

up-regulated and 13 down-regulated), the pathogenic serovar Pomona modulated 360 genes (59 up-regulated and 301 down-regulated), and the clinical isolate modulated 3,061 genes (59 up-regulated and 3,002 down-regulated) (Fig 8).

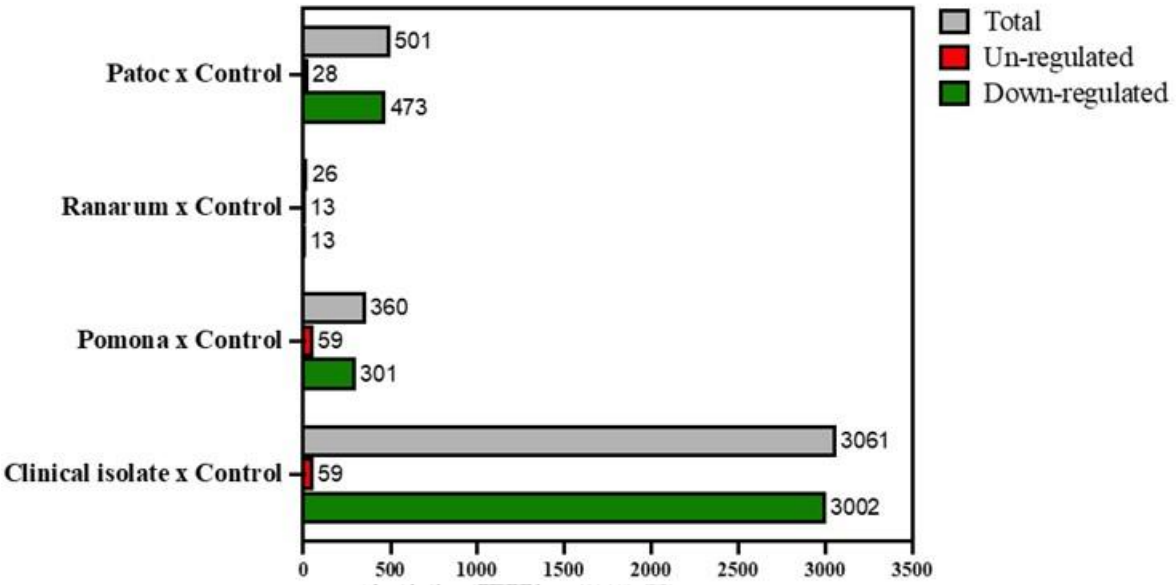


Figure 8 *Differentially expressed genes (DEGs) after 4-stimulation*

MoDCs with the non-pathogenic strains (serovar Patoc and Ranarum) and the pathogenic strains (serovar Pomona and clinical isolate) compared with unstimulated controls. The color bars showed a total of genes (grey), up-regulate (red), and down-regulated (green). The data represent three donors and FDR-adjusted is $p<0.3$, and fold change ± 1.

Principal component analysis revealed a major difference of DEGs between MoDCs stimulated with leptospires and unstimulated controls (Fig 9A). Even though the DEG genes of the non-pathogenic and the pathogenic strains were similar, the heat map (Fig 9B) showed different patterns of DEGs.

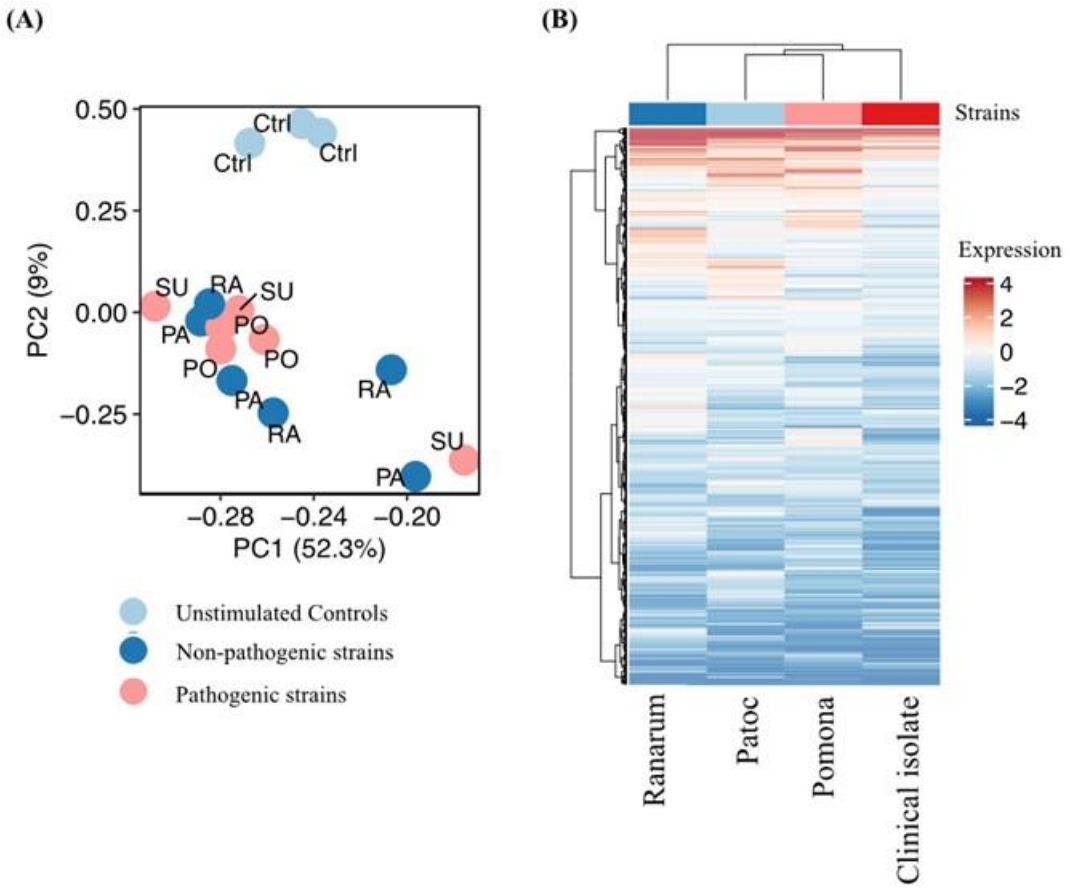


Figure 9 *Differentially expressed genes (DEGs) after 4 h-stimulation of MoDCs with the non-pathogenic strains (serovar Patoc and Ranarum), the pathogenic strains (serovar Pomona and clinical isolate), and unstimulated controls.*

(A) Principal component analysis. Light blue circles are unstimulated MoDCs used as a negative control, dark blue circles are MoDCs stimulated with non-pathogenic serovar Patoc (PA) and serovar Ranarum (RA), and pink circles are MoDCs stimulated with pathogenic serovar Pomona (Po) and clinical isolate (SU). (B) The heat map of transcriptome profiling data of the non- pathogenic and the pathogenic strains compared with unstimulated controls. The data represent three donors and FDR-adjusted is p<0.3, and fold change ± 1.

Venn diagrams show the number of modulated genes of MoDCs stimulated by leptospires in comparison with unstimulated controls (Fig 10 and Fig 11). The up-regulated genes of the two non-pathogenic strains and the two pathogenic strains

shared 10 genes and 24 genes, respectively (Fig 10A, 10B). The 24 genes found in both strains of either group were subsequently classified into genes that were common or unique to each group (Fig 10 and S7 Table 1). The results found that the non-pathogenic and the pathogenic strains upregulated the same 4 genes of MoDCs: PTX3, PELI1, IFNL1, and IFIT1. These genes are present in the JAK-STAT signaling pathway, and the biological processes involved in innate immune responses and the cytokine-mediated signaling pathway. The pathogenic strains modulated 20 unique genes of MoDCs: TNFSF9, BTG1, ISG20, NCS1, IL2RA, HERC5, KCNA3, APOBEC3A, USP18, PTGS2, RND1, BCL2A1, CRLF2, MCOLN2, EREG, IL15RA, CCL3, SOD2, RHBDF2, and EZH2. These genes involve in the cytokine-cytokine receptor interaction, the JAK-STAT signaling pathway, and the NF-Kappa B signaling pathway, the regulation of T cell tolerance induction, the regulation of inflammatory stimuli, the cytokine-mediated signaling pathway, and the regulation of defense response in biological processes. In contrast, the non-pathogenic strains upregulated 6 unique genes of MoDCs (MT2A, ISG15, CKB, CCL19, IL1B, CYP27B1), which are present in the RIG-I-like receptor signaling pathway, the NF-kappa B signaling pathway, and the chemokine signaling pathway. The biological processes of these genes are involved in interferon-γ response, mature conventional dendritic cell differentiation, the establishment of T cell polarity, and positive regulation of antigen processing and presentation.

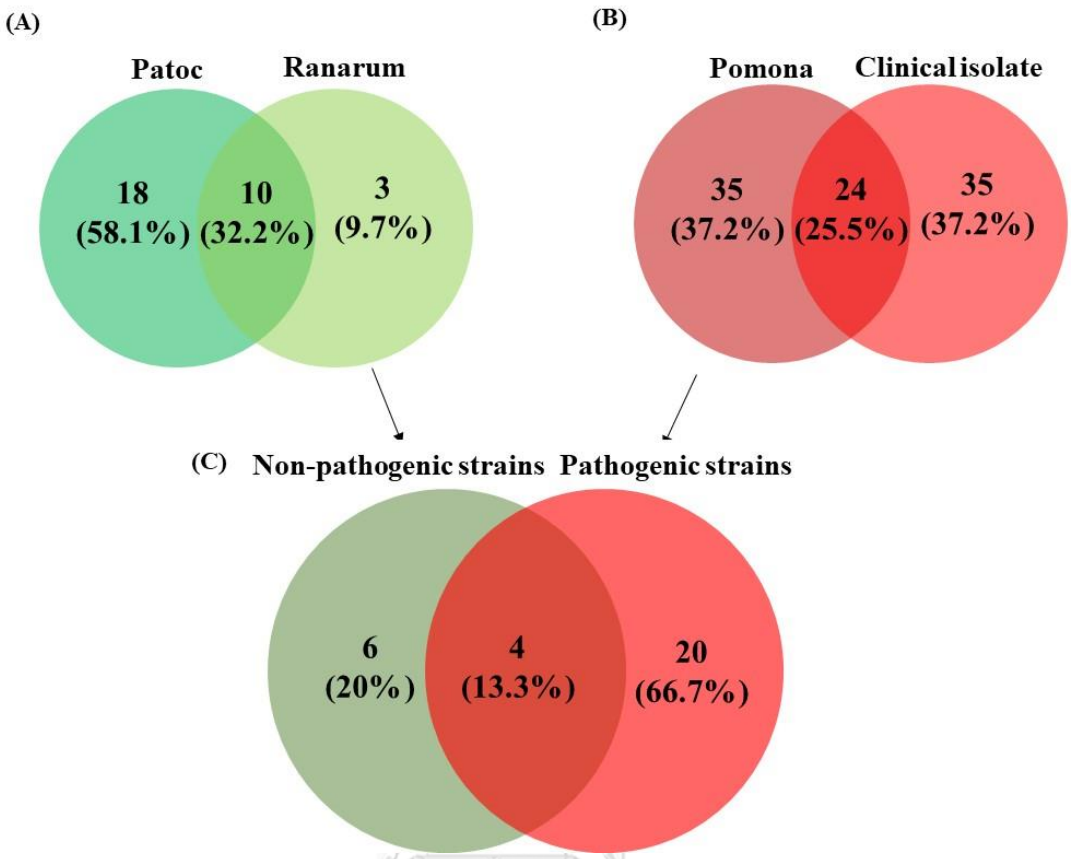


Figure 10 *Venn diagrams showing the up-regulated genes of differentially expressed genes (DEGs) after 4-h stimulation MoDCs*

(A) MoDCs with the non-pathogenic strains (serovar Patoc and Ranarum) and (B) the pathogenic strains (serovar Pomona and clinical isolate). (C) The common up-regulated genes of MoDCs stimulated with both strains of the non-pathogenic and the pathogenic leptospires.

The common down-regulated genes of MoDCs stimulated with the non-pathogenic and the pathogenic strains were 5 genes: CD180, P2RY11, SUCNR1, MAF, and APBB1IP, which are involved in the positive regulation of the LPS-mediated signaling pathway in biological processes (Fig 11A and 11B). Interestingly, 9 genes (ARPC1B, ILK, SEPTING, VCL, CRKL, MYC, ABL1, EIF4EPB1, and AKT1) from unique down-regulated genes of MoDCs stimulated with the pathogenic strains are present in the bacterial invasion of epithelial cells and the ErbB signaling pathway, and biological processes involved in the positive regulation of the LPS-mediated signaling pathway,

the B-cell activation involved in immune response (Fig 11C). In contrast, down-regulated genes of MoDCs stimulated with the non-pathogenic strains were not involved in the immune response.

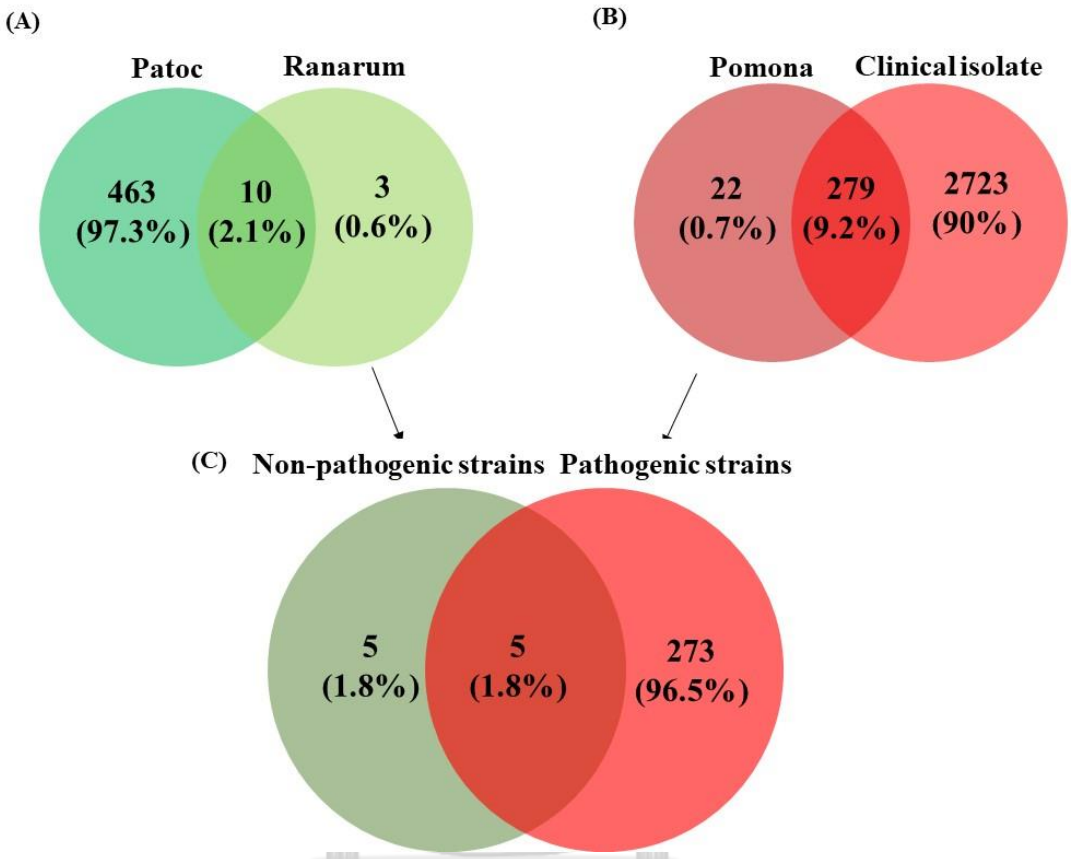


Figure 11 *Venn diagrams showing the down-regulated genes of differentially expressed genes (DEGs) after 4-h stimulation*

MoDCs with (A) the non-pathogenic strains (serovar Patoc and Ranarum), and (B) the pathogenic strains (serovar Pomona and clinical isolate). (C) The common down-regulated genes of MoDCs stimulated with both strains of the non-pathogenic and the pathogenic leptospires.

Discussion

Humans are accidental hosts and susceptible to infection with pathogenic leptospires. The main route of infection is through skin abrasions and mucous membranes where leptospires possibly encounter conventional dendritic cells (cDCs). Then, leptospires disseminate in the bloodstream and expose to immune cells such

as macrophages, monocytes and neutrophils. Circulating monocytes can infiltrate mucosal or inflamed tissues where they can differentiate into dendritic cells, called monocyte-derived dendritic cells (MoDCs). MoDCs are inflammatory dendritic cells that are found only at the sites of infection and become prominent during inflammation, in contrast to cDCs, which appear in the steady-state (48, 49). However, MoDCs and cDCs play a synergistic role in delivery of antigens to lymphoid organs during infection or inflammation. (50). Moreover, the phenotype and functions of MoDCs are similar to cDCs. Therefore, MoDCs play an important role in innate immune defense against infection and linking innate to adaptive immunity (49). This study aimed to compare the responses of human MoDCs to the non-pathogenic leptospires (*Leptospira biflexa* serovar Patoc and *L. meyeri* serovar Ranarum) and the pathogenic leptospires (*L. interrogans* serovar Pomona and a clinical isolate of *L. interrogans*) to better understand the role of MoDCs in leptospiral infection in humans, which are susceptible hosts to pathogenic *Leptospira*.

Human peripheral blood monocytes (90% purity of $CD14^+$ cells) from five donors were generated and differentiated into immature MoDCs (90% purity of $CD1a^+$ cells) (Fig 4). The cells were evaluated for their functional and phenotypic activity. First, the phagocytic activity of immature MoDCs against leptospires was determined at MOI of 100 after 10-min incubation. Immature MoDCs phagocytosed serovars Ranarum, Pomona, and clinical isolate at approximately 10% but serovar Patoc at about 5% (Fig 1 and Fig 2). This result was similar to the level of phagocytosis of immature MoDCs against *E. coli*, which was 11.5% at 30 min (51). Limited phagocytic activity of immature MoDCs may be because they are not mainly involved in immediate pathogen clearance. The phagocytosis capacity of immature MoDCs was lower than that of other phagocytic cells such as macrophages and monocytes (51). In addition, the phagocytic activity of immature MoDCs was measured at one time point after brief incubation period, which might be at the beginning of MoDC phagocytosis and activation. Immature MoDCs immediately capture antigen within 1 h and then mature to present antigen to T cells within 24 h (52, 53). Similarly, other spirochetes such as *Borrelia burgdorferi* and *Treponema pallidum* were able to activate phagocytosis of MoDCs from 1 to 24 h (54, 55). *B. burgdorferi* activated immature

MoDCs to phagocytose within 10 min and readily detected small fragments of bacteria within the MoDC cytoplasm after 1 h (55). It is possible that the phagocytosis index of immature MoDCs against leptospires might vary if we determine at different time points.

The pathogenic strains induced significantly higher apoptosis (Fig 3) , transcriptional levels of genes encoding CCL3 and TNFSF9 (S Table1), and IL-6 production (Fig.5) of mature MoDCs than the non-pathogenic strains (Fig 3 and Fig 5). *L. interrogans* has recently been reported to induce pro-inflammatory cytokines such as IL-1b, IL-6, and TNFs in severe leptospirosis, leading to cytokine storm or sepsis syndrome (6). After infection, MoDCs are generally recruited into inflammatory tissues and produce chemokines (CCL3, CCL19, CXCL14, and CXCL12) to trigger a second wave of cell recruitment (56). CCL3 or macrophage inflammatory protein-1-alpha (MIP-1α) is involved in the acute inflammatory state in the recruitment of polymorphonuclear leukocytes (57). In addition, the pathogenic strains induced apoptosis of MoDCs might be correlated with up-regulation of TNFSF9 (tumor necrosis factor superfamily ligand 9), which is known to be associated with apoptosis by activation of TLR via caspase-3, -6, and -7 (58). Moreover, previous studies demonstrated that severe leptospirosis in a sepsis state showed a loss of dendritic cells by apoptosis (59). Therefore, based on higher induction of apoptosis, the pathogenic leptospires may induce MoDC apoptosis, which may be one strategy to impair MoDC responses. Therefore, the result suggests that the pathogenesis pathway of pathogenic strains was associated with the apoptosis pathway and regulation of T cell tolerance induction, which results in leptospirosis. On the other hand, non-pathogenic strains were related to dendritic cell maturation and Th1 regulation.

The pathogenic strains stimulated immature MoDCs to differentiate into significantly lower mature MoDCs than the non-pathogenic strains as indicated by a decrease in the level of expression of CD83, a specific surface maker of mature MoDCs, and CD80 co-stimulatory molecules. The RNA interference-mediated knockdown of CD83 in human DCs reduced the capacity of DCs to stimulate T cells in an allogeneic mixed lymphocyte response. CD80 is a costimulatory molecule that binds to CD28 on

$CD4^+$ T cells. As CD80 decreased, the binding of the CD80-CD28 costimulatory pathway was weakened, possibly resulting in CD80 being involved in the $CD25^+$Treg function (60-62).

In previous studies (12), heat-killed *Leptospira* serovar Pyrogenes was used to stimulate MoDC maturation (12). In contrast, viable *Leptospira* were used in this study. Because previous report showed that viable leptospires had higher potency to activate immune cells than heat-killed ones (63). It is possible that some *in vivo* expressed protein (64-66). For example, leptospiral immunoglobulin (Lig) B protein was highly upregulated in the blood of infected host (66). In addition, the study expected that the result of MoDC response to viable pathogenic leptospires would be similar to a natural infection.than those using killed leptospires.

Based on the RNA sequencing results, IL-15RA and IL-2RA genes were up-regulated in MoDCs stimulated with the pathogenic strains. Previous reports showed that expression of IL-2RA (IL-2 receptor alpha chain, also known as CD25) was related to the induction of regulatory dendritic cells rather than stimulatory dendritic cells (67). As a result, the pathogenic leptospires-stimulated MoDCs might reduce $CD4^+$ T cell proliferation, leading to activation of Treg response. This result is similar to the immunosuppressive state of sepsis in which Tregs are involved in impairment of innate and adaptive immunity (68). However, dendritic cells becoming regulatory dendritic cells depends also on environmental conditions such as stimulation by certain cytokines (IL-10, TGF-beta) and co-stimulatory molecules (69). Our result showed that the pathogenic leptospires stimulated MoDCs to produce lower IL-10 but lower expression CD80 and CD83 than the non-pathogenic strains. (Fig 4 and Fig 5). Therefore, the pathogenic strains might impair DC maturation and induced IL-10-producing $CD4^+$ T cells, leading to Treg activation which suppresses effector T cells. Impairment of DC function might contribute to survival and dissemination of pathogenic leptospires inside the host.

In contrast, the non-pathogenic strains stimulated MoDCs with up-regulated CCL19. CCL19 generally involves the migration of MoDCs within lymph nodes (56). CCL19 is associated with enhancing the T-cell-stimulating function of mature DCs through upregulation of co-stimulatory molecules and induction of proinflammatory

cytokine production such as IL-1B and IL-12 (70). Moreover, the non-pathogenic strains increased expression of ISG15 in MoDCs. ISG15 is a ubiquitin-like protein expressed in response to IFN signaling, which is a key factor in host responses to bacterial infection (71). In this study, MoDCs stimulated with the non-pathogenic leptospires induced $CD4^+$ T cells to produce IFN-γ, which is Th1-type cytokine. Therefore, the non-pathogenic leptospires might stimulate MoDC maturation and activate Th1 to help bacterial clearance.

Based on the results in this study, we proposed the possible role of MoDCs in leptospiral infection as shown in Fig 12. Upon bacterial entry, MoDCs are recruited to infection sites and engulf leptospires. MoDCs infected the pathogenic strains subsequently produce pro-inflammatory cytokines together with other immune cells such as macrophages and neutrophils, probably leading to cytokine storm in severe leptospirosis. Moreover, MoDCs produce chemokines to mobilize immune cells. Conversely, the pathogenic strains induce apoptosis and impaired the maturation of MoDCs. In adaptive immune responses, MoDC impairment induced IL-10-producing $CD4^+$ T cells to induce Treg cells. Then, Treg cells migrate to inflammatory sites and inhibit Th1 function, leading to immune suppression. Therefore, pathogenic leptospires are able to evade host immunity and disseminate to target organs.

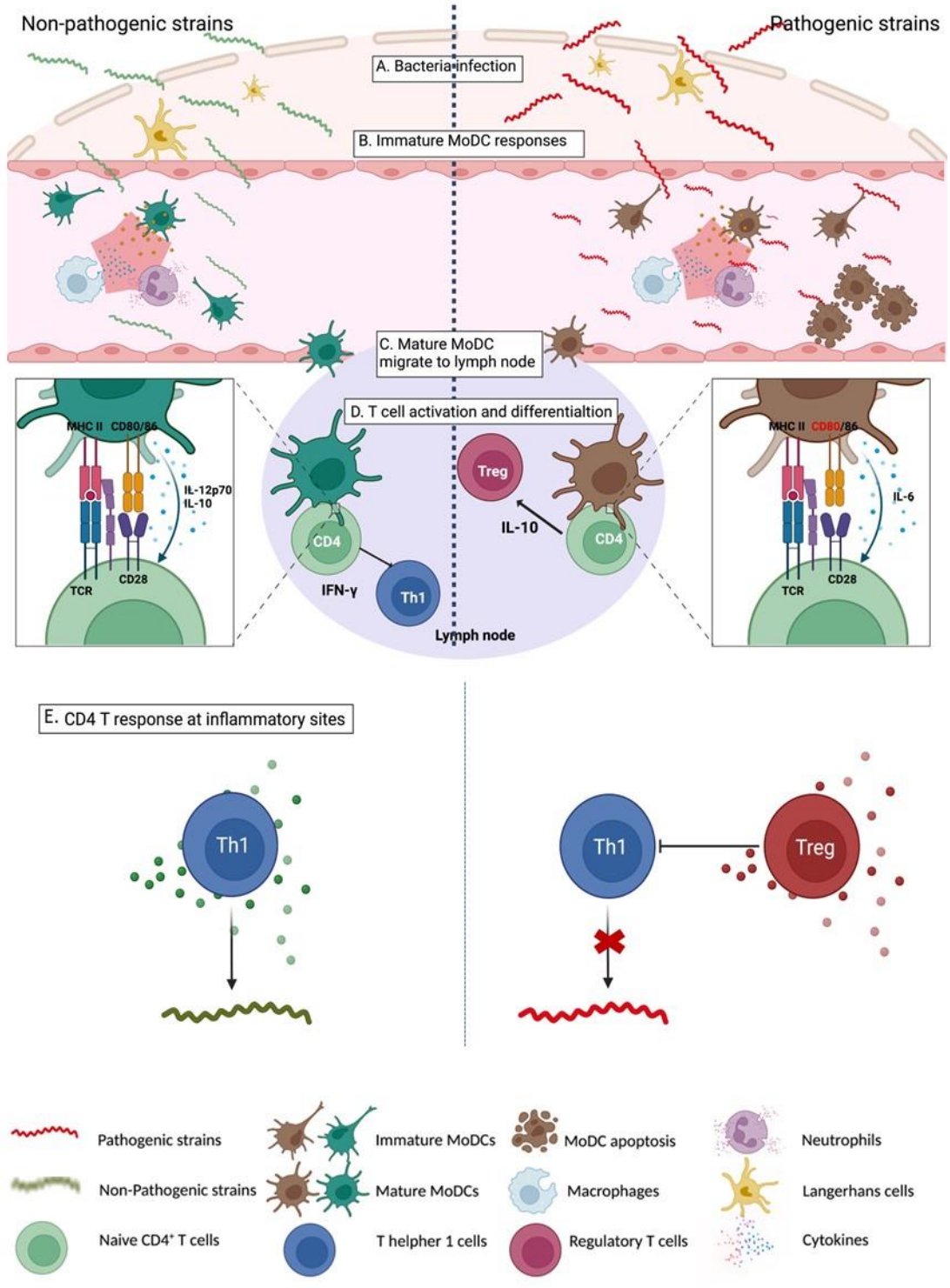


Figure 12 Schematic view of the response of human MoDCs to the pathogenic and non-pathogenic strains of leptospires.

(A) Leptospires infection through skin abrasion and the mucous membrane. (B) Immature MoDCs are recruited to the infection site. The pathogenic strains can induce

certain degree of MoDC apoptosis. (C) Mature MoDCs activate and differentiate naïve CD4+ T cells. (E) Mature MoDCs stimulated by the pathogenic strains have low CD80 and CD83, leading to may induced Treg cells. In contrast, the non-pathogenic strains activate maturation of MoDCs, leading to the may induced Th1 cells.

This study has some limitations. First, our study only focused on MoDCs, which the results might be different from other types of DCs such as cDC1, cDC2. Thus, MoDC functions should not be extrapolated to cDC (42, 72). Second, we investigated only three cytokines (IL-12p70, IL-6, and IL-10) produced by MoDCs. However, other cytokines related to severe leptospirosis such as TNF-a, IL-2, IL-4, and IL-10 (6, 73) should be further investigated. Third, we used only 5 donors, who might have different genetic background, as sources of monocytes for MoDC differentiation. Therefore, the results of some experiments might not show statistical significance. For example, although we selected MoDCs for RNA sequencing at the time point (4-h incubation) when mature MoDCs stimulated with the pathogenic and the non-pathogenic leptospires showed significant difference in term of cytokine gene expression by RT-PCR, only small number of DEGs were obtained by RNA sequencing analysis using P-value < 0.05. As a result, we had to adjust P-value to 0.3 to obtain more DEGs that might be associated with host response to leptospires including those previously shown significant results by RT-PCR. We plan to validate expression of more genes found in the transcriptomics results to in further study.

According to MoDC ability to regulate immune response, knowledge obtained from this study might be useful in new therapies and the prevention of leptospirosis. Several studies have reported that the co-delivery of adjuvant and antigen to MoDCs is essential for priming the immune response. Co-delivery has been achieved via linking antigen to adjuvant, combining antigen to protein adjuvant, or co-encapsulation in particles to enhance protective immunity (74, 75). Additionally, MoDCs have been used as vectors for vaccination against several bacterial infections (76). The BCG-infected DCs were used in tuberculosis, which is a potent T cell response and IFN-γ production, leading to significant protection against *Mycobacterium tuberculosis* in an aerosol infection model (77). Moreover, MoDCs plus heat-killed chlamydiae induced a Th1

response to protect against *Chlamydia trachomatis* infection (78). The *B. burgdorferi*-pulsed MoDCs induced a specific antibody response and protected mice against tick-transmitted spirochetes (79).

In conclusion, different MoDCs responses against the non-pathogenic strains (serovar Patoc and Ranarum) and the pathogenic strains (serovar Pomona and clinical isolate) of leptospires could partially determine the susceptibility of humans to leptospiral infection. The MoDC impairment and regulatory T cell response to IL-10 producing CD4+ T cells against the pathogenic strains could result in evasion of effector T cell response, resulting in inefficient elimination of pathogenic leptospiral infection. In contrast, increased MoDC maturation and Th1 response induced by the non-pathogenic strains could confer greater immunomodulatory effects, leading to bacterial clearance in the hosts.

ACKNOWLEDGMENTS

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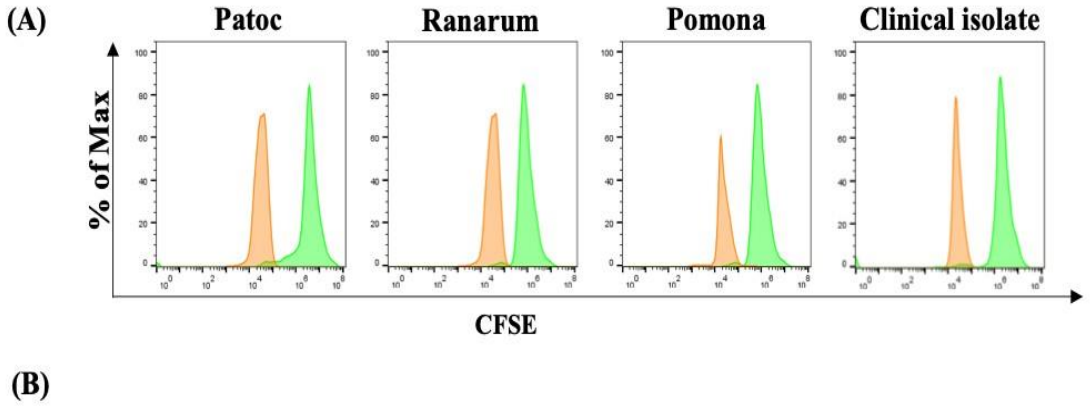
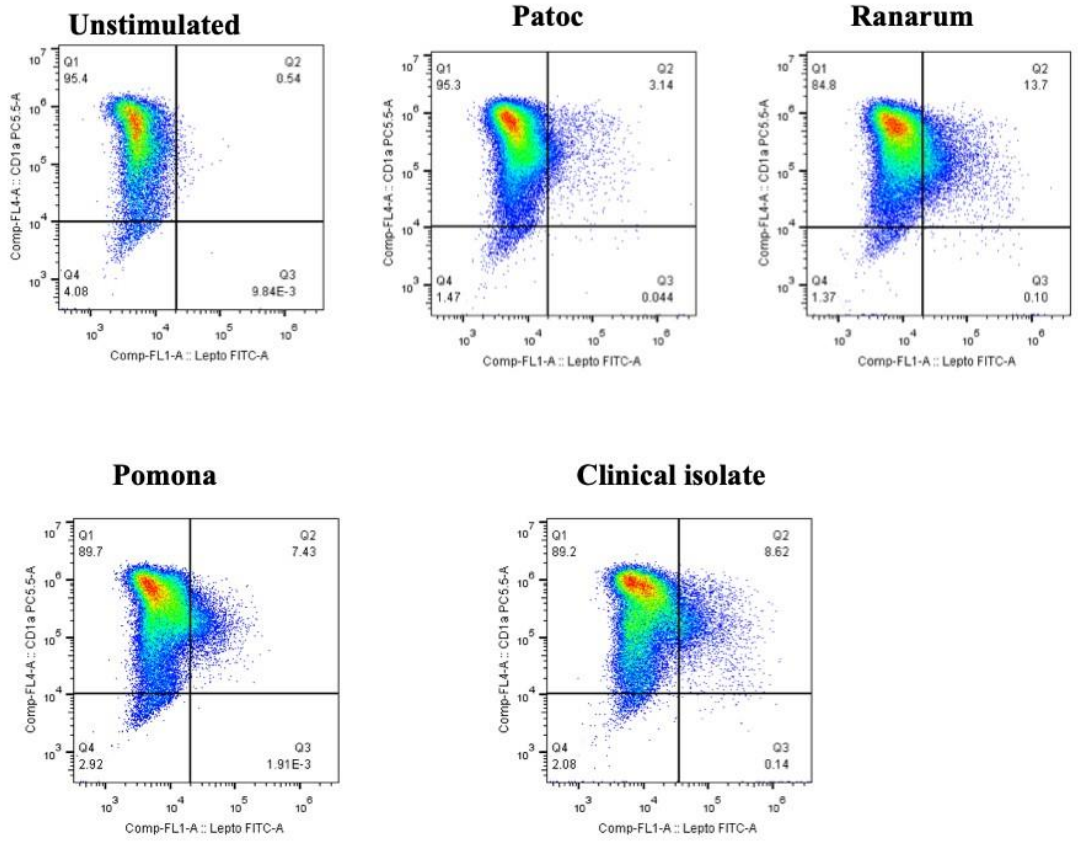
Supplementary

The supplementary Material for this article can be found online at:

Supplementary figure 1: The effect of CFSE staining with *Leptospira* was analyzed by flow cytometry.

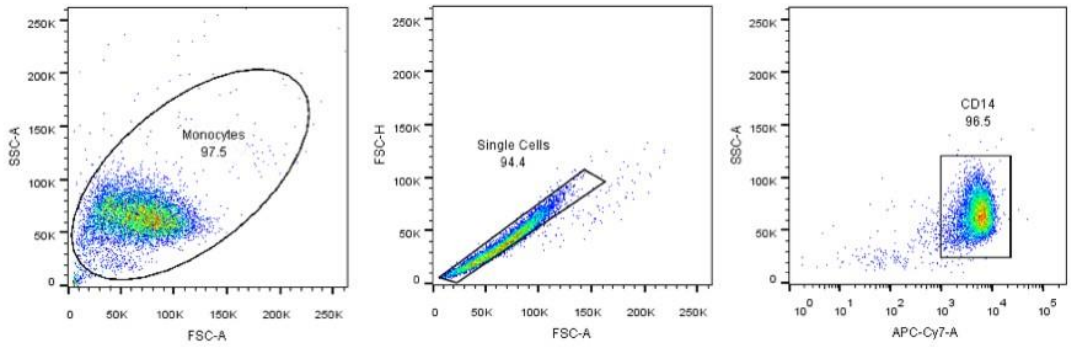


Supplementary figure 2: Fluorescence signals were measured by flow cytometry to determine the uptake of CFSE-labeled leptospires into MoDCs.

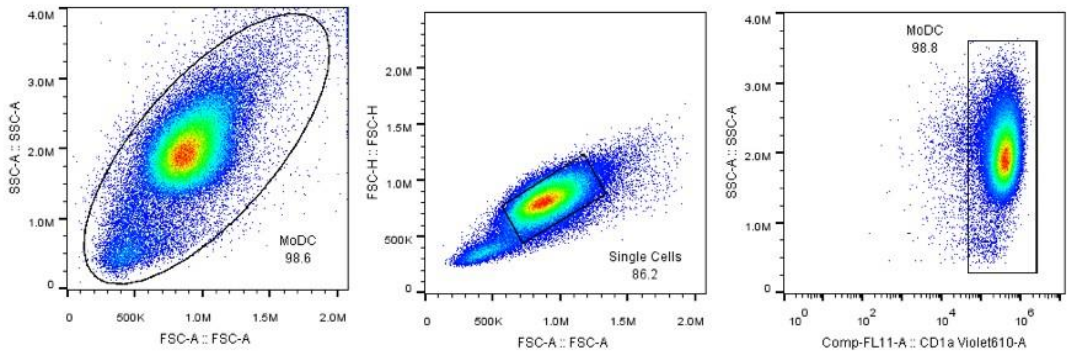


Supplementary figure 3: The purity of the monocytes ($CD14^+$) and MoDCs ($CD1a^+$) were determined by flow cytometry.

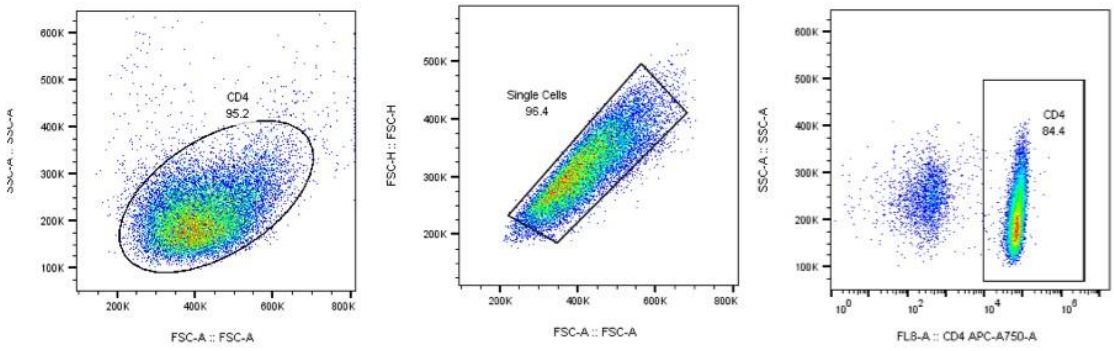
(A) The purity of the monocytes ($CD14^+$)



(B) **The purity of the MoDCs ($CD1a^+$)**



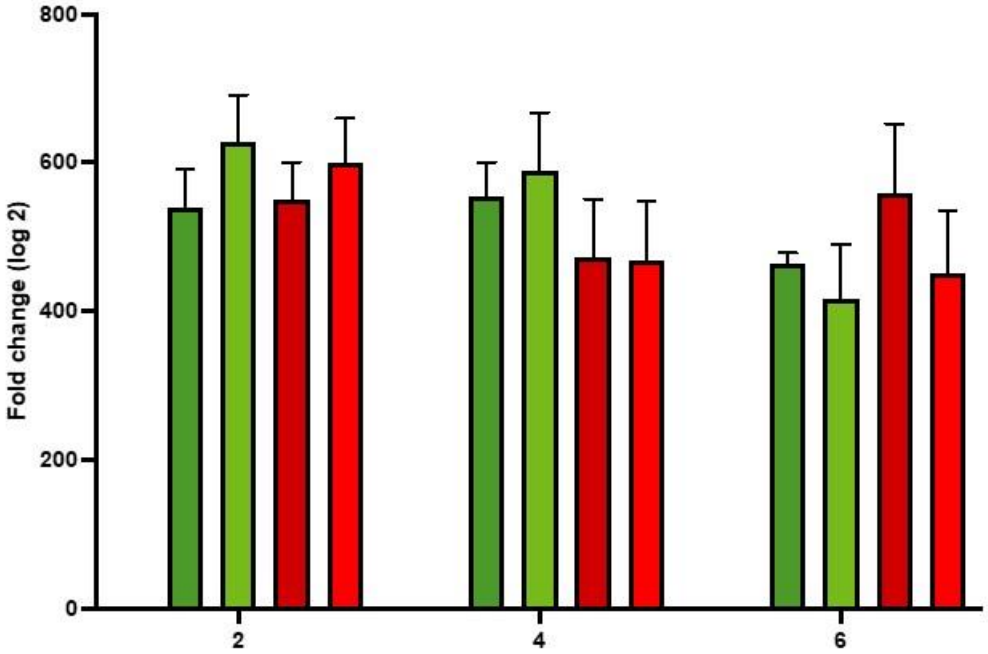
Supplementary figure 4: The purity of the $CD4^+$ T cells were determined by flow cytometry.



Supplementary figure 5: The RNA integrity number of all samples was used for transcriptome analysis.

No.	Sample Name	Donor number	Concen-tration(ng/μL)	Volume(μL)	Total Mass(μg)	RIN	28S/ 18S	Library Type	Test Result
1	1 Unstimulated	1	21.98	29	0.6374	9.1	1.2	DNBSEQ Transcriptome	Level A
2	1 Patoc	1	24.78	29	0.7186	9.3	1.2	DNBSEQ Transcriptome	Level A
3	1 Rararum	1	25.41	29	0.7369	9.4	1.3	DNBSEQ Transcriptome	Level A
4	1 Pomona	1	22.61	29	0.6557	9.1	1.2	DNBSEQ Transcriptome	Level A
5	1 Surin	1	18.54	29	0.5377	9.1	1.5	DNBSEQ Transcriptome	Level A
6	2 Unstimulated	2	42.03	29	1.2189	8.9	1.2	DNBSEQ Transcriptome	Level A
7	2 Patoc	2	30.81	29	0.8935	9.2	1.3	DNBSEQ Transcriptome	Level A
8	2 Ranarum	2	23.05	29	0.6685	9.3	1.3	DNBSEQ Transcriptome	Level A
9	2 Pomona	2	30.36	29	0.8804	9.2	1	DNBSEQ Transcriptome	Level A
10	2 Surin	2	34.64	29	1.0046	8.6	1.4	DNBSEQ Transcriptome	Level A
11	3 Unstimulated	3	30.13	29	0.8738	8.8	1.3	DNBSEQ Transcriptome	Level A
12	3 Patoc	3	25.02	29	0.7256	9.1	1.1	DNBSEQ Transcriptome	Level A
13	3 Ranarum	3	59	29	1.71	9.2	1.6	DNBSEQ Transcriptome	Level A
14	3 Pomona	3	52.7	29	1.5283	8.4	1.2	DNBSEQ Transcriptome	Level A
15	3 Surin	3	85	29	2.47	9.3	1.4	DNBSEQ Transcriptome	Level A

Supplementary figure 6: The cytokine gene expression of IL-1β in the MoDCs stimulated with the pathogenic and non-pathogenic strains at 2,4 and 6 h.



Supplementary Table 1: The up and down regulation of differential expression gene associated with top three GO terms investigated in MoDC infected with pathogenic and non-pathogenic strains of leptospires.

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
PTX3	Pentraxin	- Regulation of glycoprotein metabolic process - Negative regulation by host of viral process - Modulation by host of viral process	3.86, 0.005	3.86, 0.01	3.86, 0.02	3.86, 0.28
PELI1	Pellino E3 Ubiquitin	- Toll signaling pathway - Negative regulation of necroptotic process - Negative regulation of programmed cell death	3.76, 0.01	3.76, 0.02	3.76, 0.02	3.76, 0.07
IFNL1	Interferon Lamba 1	- Positive regulation of MHC class I biosynthetic process - Negative regulation of interleukin-13 production - Negative regulation of interleukin-5 production	3.37, 0.02	3.36, 0.07	3.37, 0.05	3.37, 0.05

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
IFIT1	Interferon Induced Protein with Tetratricopeptide Repeats1	- Regulation by virus of viral protein levels in host cell - Response to type I interferon - Regulation of helicase activity	3.59, 0.02	3.38, 0.29	3.59, 0.09	3.59, 0.04
TNFSF9	Tumor Necrosis Factor Ligand Superfamily Member 9	- Regulation of lymphocyte proliferation - Positive regulation of activated T cell proliferation - Regulation of programmed cell death	3.19, 0.02	2.49, 0.03	-	-
BTG1	B cell Translocation gene, Anti-Proliferation Factor 1	- Positive regulation of fibroblast apoptotic process - Regulation of fibroblast apoptotic process - endothelial cell differentiation -	3.35, 0.03	2.12, 0.09	-	-

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
ISG20	Interferon Stimulated Exonuclease Gene 20	- DNA catabolic process, exonucleolytic - DNA catabolic process - Nucleobase-containing compound catabolic process	3.05, 0.04	1.4, 0.26	-	-
NCS1	Neuronal Calcium Sensor 1	- Regulation of plasma membrane bounded cell projection organization - Regulation of neuron projection development	2.78, 0.04	1.41, 0.26	-	-
IL2RA	Interleukin 2 Receptor Subunit Alpha	- Regulation of T cell tolerance induction - Interleukin-2-mediated signaling pathway - Cellular response to interleukin 2	2.61, 0.04	1.47, 0.23	-	-

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
HERC5	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 5	- ISG15-protein conjugation - Regulation of defense response to virus - Regulation of response to biotic stimulus	2.21, 0.04	1.46, 0.25	-	-
KCNA3	Potassium Voltage-Gated Channel Subfamily A Membrane 3	- Potassium ion transport - Potassium ion transmembrane transport - Cation transport	2.60, 0.05	1.46, 0.21	-	-
APOBEC3A	Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3A	- DNA cytosine deamination - Cytidine to uridine edition - DNA deamination	2.92, 0.05	1.44, 0.26	-	-

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
USP18	Ubiquitin Specific Peptidase 18	- Negative regulation of type I interferon-mediated signaling pathway - Regulation of type I interferon-mediated signaling 1pathway - Negative regulation of innate immune response	2.48, 0.05	1.46, 0.26	-	-
PTGS2	Prostaglandin-Endoperoxide Synthase 2	- Regulation of fever generation - Positive regulation of prostaglandin biosynthetic process - Positive regulation of heat generation	1.67, 0.06	1.46, 0.23	-	-
RND1	Rho Family GTPase 1	- Negative regulation of cell adhesion - Regulation of actin filament-based process - Regulation of actin cytoskeleton organization	2.13, 0.06	1.47, 0.23	-	-

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
BCL2A1	BCL2 Related Protein A1	- Extrinsic apoptotic signaling pathway in absence of ligand - Intrinsic apoptotic signaling pathway in response to DNA damage - Extrinsic apoptotic signaling pathway	2.74, 0.07	1.46, 0.28	-	-
CRLF2	Cytokine Receptor like Factor 2	- Positive regulation of mast cell activation - Positive regulation of interleukin 5 production - Regulation of receptor signaling pathway via STAT	2.70, 0.09	1.46, 0.22	-	-
MCOLN2	Mucolipin TRP Cation Channel 2	- Calcium ion transmembrane transport - Calcium ion transport - Inorganic cation transmembrane transport	2.06, 0.09	1.47, 0.22	-	-

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
EREG	Epiregulin	- Clatherin-coated endocytic vesicle memvbrane - Receptor ligand activity - mRNA transcription	1.96, 0.14	1.37, 0.22	-	-
IL15RA	Interleukin 15 Receptor Subunit Alpha	- Cellular response to interleukin 15 - Positive regulation of phagocytosis - Cytokine-mediated signaling pathway	2.47, 0.16	1.38, 0.21	-	-
CCL3	C-C Motif Chemokine Ligand 3	- CCR1 chemokine receptor binding - CCR5 chemokine receptor binding - Positive regulation of natural killer cell chemotaxis	4.48, 0.18	1.73, 0.24	-	-

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
SOD2	Superoxide Dismutase 2	- Positive regulation of vascular associated smooth muscle cell apoptotic process - Oxygen homeostasis - Positive regulation of vascular associated smooth muscle cell differentiation	4.14, 0.21	1.90, 0.19	-	-
RHBDF2	Rhomboid 5 Homolog 2	- Regulation of ERBB signaling pathway - Negative regulation of protein transport - Negative regulation of secretion by cell	2.22, 0.25	1.41, 0.20	-	-
EZH2	Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit	- Positive regulation of cell cycle phase transition - Histone methylation - Negative regulation of gene expression, epigenetic	1.80, 0.29	1.27, 0.22	-	-

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
MT2A	Metallothionein 2A	- Cellular response to erythropoietin - Cellular copper ion homeostasis - Intracellular membrane-bounded organelle	-	-	3.83, 0.02	3.83, 0.01
ISG15	ISG15 Ubiquitin Like Modifer	- ISG15-protein conjugation - Response to type I interferon - Regulation of type I interferon-mediated signaling pathway	-	-	3.90, 0.02	3.90, 0.01
CKB	Creatine Kinase B	- Creatine metabolic process - Creatine kinase activity - Phosphotransferase activity, nitrogenous group as acceptor	-	-	2.78, 0.03	2.78, 0.11

Gene symbol	Gene name	GO annotation	Average fold changes and Adj P. values			
			Pathogenic strains		Non-pathogenic strains	
			Serovar Pomona	Clinical isolate	Serovar Patoc	Serovar Ranarum
CCL19	C-C Motif Chemokine Ligand 19	- Establish of T cell polarity - Positive regulation of antigen processing and presentation - Regulation of T-helper 1 cell differentiation	-	-	2.96, 0.06	2.96, 0.11
IL1B	Four-Jointed Box Kinase 1	- Regulation of T-helper 1 cell cytokine production - Positive regulation of T-helper 1 cell cytokine production - Regulation of fever generation	-	-	2.50, 0.14	2.50, 0.28
CYP27B1	Cytochrome P450 Family 27 Subfamily B Member 1	- Mitochondrial outer membrane	-	-	3.05, 0.19	3.05, 0.28

Part II: Sublethal infection of C3H/HeNJ against *Leptospira interrogans* serovar Pomona

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Abstract

Leptospirosis is a worldwide zoonotic disease caused by pathogenic *Leptospira* spp. Leptospires can infect a variety of mammalian species. Golden Syrian hamsters are mostly used to study acute leptospirosis. However, the immunopathogenic mechanism is poorly understood because immunological reagents for hamsters are limited. This study aimed to establish C3H/HeNJ mice as an animal model for leptospirosis. Five-week-old C3H/HeNJ mice were infected with either low (10^3 cells) or high (10^6 cells) inoculum dose of *Leptospira interrogans* serovar Pomona. All mice were investigated for survival rate, leptospiral load and histopathology of target organs, antibody levels, and cytokine production (IFN-γ, IL-6 and IL-10) at day 28 post-infection. All infected mice survived and did not develop clinical signs of leptospirosis. However, C3H/HeNJ mice infected with 10^6 cells of leptospires showed kidney colonization of leptospires and pathological changes in the lung and kidney including renal fibrosis. The glomerular size in PAS-D stained kidney tissues of C3H/HeNJ mice infected with 10^6 cells of leptospires was significantly reduced compared to that of mice infected with 10^3 cells of leptospires and non-infected mice. 10^6 leptospiral cells induced significantly higher levels of IFN-gamma and IL-6 than 10^3 leptospira cells, but IL-10 level was not significantly different. Moreover, 10^6 leptospiral cells induced predominant IgG2a isotype suggesting Th1-like response. These results suggest that C3H/HeNJ mice may be used as a sublethal model of leptospirosis.

Keywords: *Leptospira*, Leptospirosis, C3H/HeNJ, sublethal infection, mouse model, kidney fibrosis

Introduction

Leptospirosis, a globally important zoonosis, is caused by pathogenic Leptospira species. Tropical and sub-tropical regions are endemic areas of the disease (80). A wide variety of animals are carriers of Leptospira infection, but rodents especially rats are recognized as important reservoirs hosts (81, 82). *Leptospira* can colonize and persist in renal tubules of reservoirs, from which they are shed into urine to contaminate surrounding environments (31) Leptospires commonly transmit to humans through skin abrasion and mucous membrane after exposure to urine of infected animals or contaminated soil and water in rural area and urban slum communities. Approximately 1.03 million human leptospirosis cases and 58,900 deaths were reported annually worldwide (83). In Thailand, the mortality rate of leptospirosis was reported to be 0.09 per 100,000 population (84)

The pathogenic mechanism of leptospirosis is not clearly understood (1) Most in vivo studies focused on pathophysiology of leptospiral infection, zoonotic cycle of leptospirosis, and protective efficacy of vaccine against Leptospira infection (82) while host immune response against *Leptospira* infection is yet limited. Guinea pigs and hamsters have been used as animal models for acute leptospirosis, especially for studies on virulence factors and vaccine efficacy because their clinical outcomes resemble those of acute human leptospirosis (85). Nonetheless, the lack of immunological reagents for use in hamsters and guinea pigs has limited an understanding of immune response to Leptospira. Rats and mice are natural reservoir hosts of leptospires and commonly used as chronic leptospirosis models. However, mice presented a wide range of clinical outcomes including lethal, sublethal and chronic leptospirosis depending on several factors such as strain, genetic background, age, and sex of tested animals, dose and route of infection (82) For example, after intraperitoneally infection with 10^6 cells of *L. interrogans* serovar Manila, 9-week-old C3H/HeJ mice survived more than 28 days and showed symptoms of chronic leptospirosis (86). However, C3H/HeJ mice died with acute leptospirosis on day 4 when 100-fold increase in inoculum dose was used. In another study, at the same dose (10^7 cells) of *L. interrogans* serovar Copenhageni, 3-week-old of C3H/HeJ mice died of acute

leptospirosis within 5 days, while 6-week-old mice survived up to 7 days and presented sublethal symptoms (37)

The challenge with *L. interrogans* serovar Icterohaemorraghiae caused mortality in C3H/HeJ mice but not in C3H/HeN mice (24). Although they are closely related strains, C3H/HeJ mice are characterized by hyporesponsiveness to lipopolysaccharide (LPS) because they have a single point mutation of LPS allele (Lps^d) in the gene encoding Toll-like receptor 4 (TLR4) compared to the C3H/HeN mice (87, 88) These revealed the crucial effects of TLR4 on leptospirosis pathogenesis. The disadvantages of C3H/HeJ mouse model are that it is considered slightly immunocompromised (89) and rather expensive. C3H/HeNJ mice, a result of hybridization mating between C3H/HeJ (TLR4 defective mice) and C3H/HeN (wild-type TLR4) mice, are commercially available at reasonable price. However, the use of C3H/HeNJ mice as an animal model for leptospirosis has not been reported. Therefore, this study aimed to investigate the susceptibility of C3H/HeNJ mice against *Leptospira interrogans* serovar Pomona that was previously shown to be highly virulent in hamsters (90).

Materials and methods

Animals and ethics statement

Inbred female C3H/HeNJ mice at 4 weeks old were purchased from Nomura Siam International and outbred female Syrian golden hamsters at 4 weeks old were purchased from the Animal Laboratory Breeding Unit, Faculty of Medicine, Khon Kaen University, Thailand. Each animal was housed in separate cage in an ABSL-2 pathogen-free environment in the animal research laboratory of the Armed Forced Research Institute of Medical Sciences (AFRIMS), Thailand. The mice and hamsters were quarantined for 7 days before inoculation.

The protocols for animal manipulation were approved by the Institutional Animal Care and Use Committee (IACUC) of AFRIMS (approval number: ARC 2/2563). The protocols followed Thai National Animals for Scientific Purposes Act, BE 2558 (AD 2015), and were conducted under licenses issued by the Institute for Animals for Scientific Purpose Development and National Research Council of Thailand.

Bacterial strains and culture

L. interrogans serovar Pomona was directly cultured and isolated from kidneys of infected hamsters in our previous experiment (90) followed by less than three in vitro passages. Leptospires were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (BD Difco™) supplemented with 10% bovine serum albumin (BSA) solution (Zuerner, 2005) at 30 °C until the log phase was reached (2×10^8 cells/ml). Leptospires were enumerated using Petroff-Hausser chamber under a dark-field microscope.

C3H/HeNJ genotyping

Approximately 3 mm of small tail clip was collected from mice under anesthesia with isoflurane. The genomic DNA was extracted by using TissueLyser LT (Qiagen) with QIAamp Fast DNA Tissue Kit (Qiagen) according to the manufacturer's protocols. The 717-bp of TLR4 region was amplified using Q5® High-Fidelity DNA polymerase (NEB). The specific primer pairs including forward primer (5'-GCAGTGGGTCAAGGAACAGA-3') and reverse primer (5'-TCCTCTGCTGTTTGCTCAGG-3') were designed based on the mRNA sequence of TLR4 gene (NCBI reference sequence: AF110133.1). The amplified product was purified using GeneJeT Gel Extraction (Thermo Scientific) according to the manufacturer's protocols and verified by DNA sequencing (Macrogen Inc.). The TLR4 sequences of C3H/HeN mice (AF177767.1) and C3H/HeJ mice (AF110133.1) were aligned and compared using the BioEdit program.

Leptospiral infection in C3H/HeNJ mice

C3H/HeNJ mice (N=3-5 per group) were intraperitoneally injected with 100 µl of 10^3 cells (low inoculum dose), 10^6 cells (high inoculum dose) of leptospires, or sterile phosphate buffered saline (PBS). Hamsters (N=5 per group) were intraperitoneally injected with 10^3 cells of leptospires. The injected animals were monitored daily for clinical signs of acute leptospirosis (Conrad et al., 2017; Coutinho et al., 2011). The animals that reached any endpoint criteria or survived up to 28 days were euthanized with an overdose of isoflurane.

Blood was collected by cardiac puncture. Kidney, lung, and liver were collected and then preserved in 10% neutral buffered formalin for histopathology and RNAlater™ Stabilization Solution (Thermo Scientific) for leptospiral DNA detection. Spleen was collected and maintained in RPMI 1640 (Gibco) containing 100 unit/ml penicillin and 50 µg/ml streptomycin (Gibco) at 4 °C for cytokine detection.

Leptospira detection

A hundred microliter of blood samples were inoculated into semisolid EMJH medium (0.2% agar). Kidney, lung, and liver samples were sliced, pulverized by passing through 5 ml syringe, and inoculated into semisolid EMJH medium. The cultures were serially diluted 10-fold to 100-fold before incubation. The cultures were observed weekly for actively motile leptospires under a dark-field microscope.

Total genomic DNA was extracted from approximately 30 mg of kidney tissue using TissueLyser LT (Qiagen) and QIAamp Fast DNA Tissue Kit (Qiagen). The leptospiral genome was detected using QuantStudio 5 Real-Time PCR System (Applied Biosystem) combined with SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) and lipL32-specific primers (91) according to manufacturer's instructions. The cycle threshold (Ct) values of all samples were compared to leptospiral DNA standard curve constructed by using previous protocol (90) to calculate leptospiral load.

Antibody detection

Antibody titers were measured by enzyme-linked immunosorbent assay (ELISA) following the previous protocol (90). Each well was coated with 100 µl of lysate of heat-killed leptospires (5 µg/ml) or BSA (5 µg/ml). Sera were diluted with PBST (PBS plus Tween 20 at a final concentration of 0.05%) at a dilution of 1:100-1:312,500 before use as a primary antibody. Horseradish peroxidase (HRP) -conjugated goat anti-mouse antibody (KPL, diluted 1:5000) was used as a secondary antibody. The detection was performed using a 3,3',5,5' tetramethylbenzidine (TMB) Substrate Reagent Set (BD bioscience). Midpoint titers were determined using S-curve method.

Histopathology examination

Kidney, lung, and liver tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E), Periodic Acid-Schiff with diastase (PAS-D), and Masson's trichrome following the previous protocol (92). The histopathological examination was operated by a veterinary pathologist who scored in a blinded manner. The score of pulmonary hemorrhage was assessed as 0 (none), 1 (single focus), 2 (multivalent foci), or 3 (severe). Pathological change in liver was graded by inflammatory foci in 10 fields at a final magnification of 100× as 0 (none), 1 (1–3), 2 (4–7), or 3 (>7). Interstitial inflammation and tubular damage were evaluated as 0 (normal), 1 (mild), 2 (moderate), or 3 (severe). To measure the glomerulus size stained with PAS-D were evaluated in five random non-overlapping fields per sample at a final magnification of 100× under Olympus CX33 light microscope with Canoscope®5MP-DG-105-W (Nair et al., 2020b). Masson's trichrome staining of kidney tissues was used to visualize collagen deposition to determine renal fibrosis (93). All images were taken at final magnifications of 40×, 100×, or 400×.

Immunohistochemistry (IHC) staining

The IHC staining was performed following the previous protocol (94, 95) A LipL32-specific mouse monoclonal antibody (in house preparation, diluted 1:50) was used as a primary antibody and HRP-conjugated goat anti-mouse antibody (KPL, diluted 1:100) was used as a secondary antibody. The detection was performed using TMB Substrate Reagent Set, and all images were taken at a final magnification of 40×.

Cytokine detection

The spleen was diced and placed into a 70 µm cell strainer before splenocytes were isolated using a plunger end of syringe and rinsed with RPMI 1640. The splenocytes were transferred to a 15-ml conical tube and washed twice with RPMI 1640. T cell activation was performed following the previous protocol (24). A hundred microliter of 5 µg/ml soluble anti-mouse CD3 (Biolegend) were coated in each well of 96 well plates (Corning). The 50 µl of 3×10^5 cells of splenocytes were stimulated with 50 µl of either 1×10^6 cells of heat-killed *L. interrogans* serovar Pomona or PBS. The plates were incubated at 37 °C for 72 h and culture supernatant was collected. The

cytokine level in the supernatant was measured using ELISA kits of Ultra-LEAFTM Purified anti-mouse of Interferon Gamma (IFN-γ), Interleukin-6 (IL-6), and IL-10 (Biolegend) according to the manufacturer's protocols.

Statistical Analysis

All statistical analyses were performed using the GraphPad Prism software (version 9.2, Chulalongkorn University license). Survival curves were plotted as Kaplan-Meier curves and the difference was tested using a log-rank test. The difference in other parameters between groups was determined using the one-way analysis of variance (ANOVA) test. Results were considered as statistical significance when $p<0.01$.

Results

C3H/HeNJ mice carrying a homozygous wild-type TLR4 gene

The TLR4 genotype of C3H/HeNJ mice was identified by DNA sequencing using primers covering the location of point mutation on TLR4 gene (Fig. 13A). The point mutation on TLR4 gene of C3H/HeJ was found at the nucleotide 2136 where cytosine (C) is replaced with adenine (A) (87) (Fig. 13B). In contrast, the nucleotide sequences of C3H/HeNJ and C3H/HeN mice are identical indicating homozygous wild-type TLR4 gene (Fig. 13B).

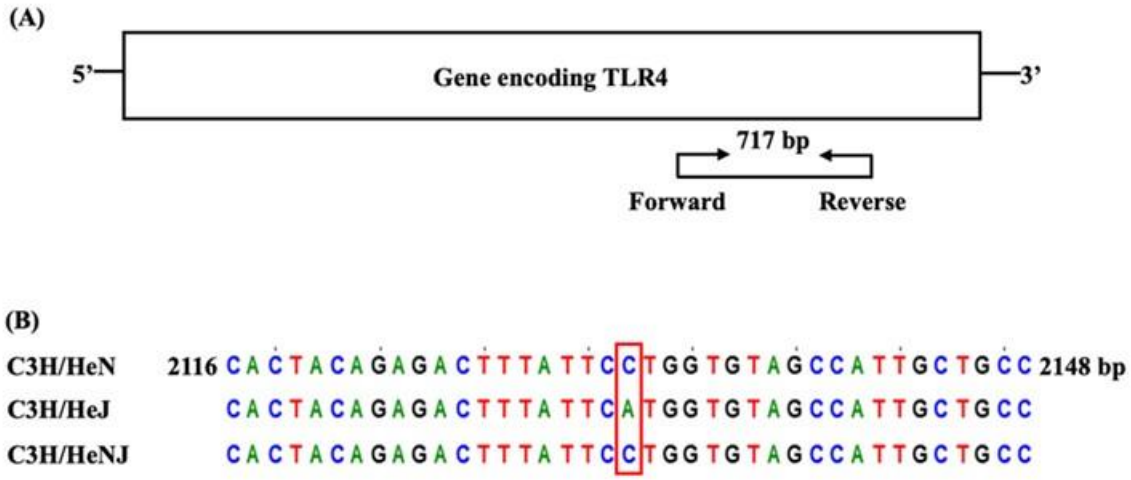

Figure 13 *Genotyping of TLR4 of C3H/HeNJ mice.*

(A) Schematic indicating primer binding locations. The primer pairs indicated by the black arrows amplified the 717-bp product containing the location of point mutation. The forward and reverse primer binding sites are nucleotides 2045-2064 and 2742-2761, respectively. (B) Pairwise alignment of TLR4 sequence. The DNA sequence of C3H/HeNJ mice is compared to the sequences of C3H/HeN mice (wild-type TLR4) and C3H/HeJ mice (natural TLR4 mutant) using the BioEdit program. The red box indicates the nucleotide 2136, where is the location of point mutation of TLR4 gene of C3H/HeJ.

Survival of C3H/HeNJ mice after L. interrogans serovar Pomona infection

C3H/HeNJ mice were infected with two different inoculum doses, either 10^3 or 10^6 cells of virulent leptospires. All infected mice survived until the end of the experiment (28 days post-infection) and did not develop any clinical signs of leptospirosis similar to the negative control group (Table 1 and Supplementary Fig. 1A). In contrast, hamsters used as a control of acute leptospirosis died within 10 days as expected. All mice gradually gained weight after infection whereas hamsters started to lose their weight after 7 days of infection and eventually died (Supplementary Fig. 2B).

Table 1 Survival rate and leptospiral culture detection in target organs of infected animals

Animals	Inoculum dose	Survival rate (%)[a]	Positive culture detection (%)[b]			
			Blood	Kidney	Lung	Liver
C3H/HeNJ mice	PBS	100 (3/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)
	10^3 cells	100 (5/5)	0 (0/5)	40 (2/5)	0 (0/5)	0 (0/5)
	10^6 cells	100 (5/5)	0 (0/5)	40 (2/5)	0 (0/5)	0 (0/5)
Hamsters	10^3 cells	0 (0/5)	100 (5/5)	20 (1/5)	40 (2/5)	40 (2/5)

[a]Parenthesis reveals the number of surviving animals/total number of tested animals.
[b]Parenthesis reveals the number of samples with positive culture detection/total number of tested samples.

Renal colonization of leptospires in infected C3H/HeNJ mice

Conventional culture and molecular detection methods were used to investigate the presence of leptospires in target organs of the infected animals. After infection with different doses, viable leptospires were detected by culture in kidneys of 2 out of 5 mice in both infected groups (Table 1). The qPCR detected leptospiral DNA in only kidneys of all infected mice but not detected in both lungs and livers. The leptospiral burden in kidneys of mice infected with 106 leptospiral cells (875.64 ± 107.08) was significantly higher than those infected with 10^3 cells (240.80 ± 83.00) of leptospires (Fig. 14A). IHC staining using LipL32-specific mouse monoclonal antibody confirmed renal colonization of leptospires in glomerulus, tubular epithelial surface, and interstitium (Fig. 14B). As expected, no leptospires was detected by both methods in PBS-treated mice. Viable leptospires were detected in blood cultures of all hamsters but only in kidney culture of one hamster (Table 1). However, the leptospiral DNA was detected in kidneys of all hamsters.

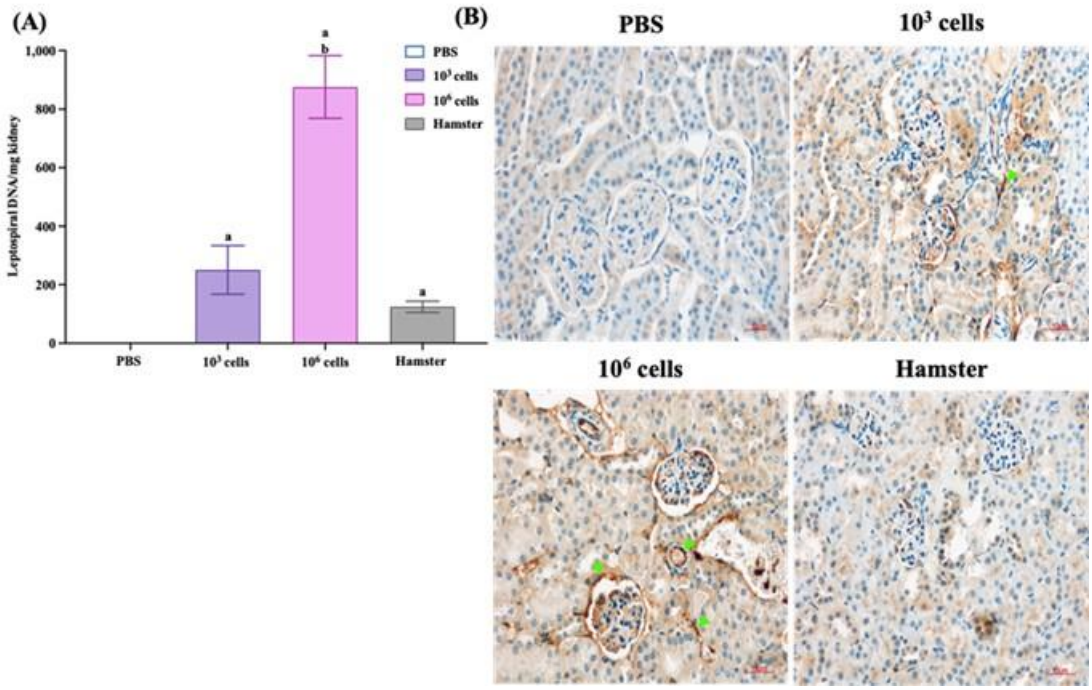

Figure 14 *Renal colonization of leptospires in C3H/HeNJ mice.*

C3H/HeNJ mice were infected with either 10^3 cells or 10^6 cells of leptospires, and PBS as a control, while hamsters were infected with 10^3 cells of leptospires. (A) Leptospiral

DNA detection by quantitative PCR. The amplification was performed using the detection of lipL32 gene in the kidneys of infected animals. Each bar represents mean leptospiral genome copies per mg kidney tissue ± standard deviation (SD). Statistical values were analyzed by Bonfferoni's multiple comparisons test. Letter a and b indicate significant differences compared to the PBS-treated group and the group infected with 103 cells of leptospires, respectively. (B) The immunohistochemistry staining using LipL32-specific antibody was performed to detect leptospires in the kidneys of infected animals. The brown color (green arrows) indicates stained leptospires.

Organ damage in C3H/HeNJ mice after leptospiral infection

Histopathological changes of kidney, lung, and liver tissues from the infected mice were scored based on severity. The interstitial nephritis showing mononuclear cell infiltration was more severe in C3H/HeNJ mice infected with 10^6 cells of leptospires than those infected with 10^3 cells of leptospires and PBS (Fig. 15 and Fig. 16). The pathological finding of lung tissues from C3H/HeNJ mice infected with 10^6 cells of leptospires illustrated mild parenchymal damage with mononuclear cell infiltration as well as alveolar spaces filled with red blood cells suggesting lung damage (Fig. 15 and Fig. 16). Even though the histology score of renal and alveolar hemorrhage was also found in C3H/HeNJ mice infected with 10^3 leptospiral cells and PBS, the severity scores were significantly lower than those infected with 10^6 cells of leptospires (Fig. 15). However, liver of all mice showed no histopathological change (Fig. 15 and Fig. 16).

Using Masson trichrome staining, kidney tissues of C3H/HeNJ mice infected with 10^6 cells of leptospires showed increased accumulation of collagen, suggesting renal fibrosis, in comparison to those infected with 10^3 cells of leptospires (Fig. 17). Moreover, the shrinkage of glomeruli was observed in C3H/HeNJ mice infected with 10^6 cells of leptospires as demonstrated by significant decrease of glomerular size compared to those infected with 10^3 cells of leptospires and PBS-treated mice (Fig. 18). However, the histopathological change of hamsters was not investigated because there was no survival of hamsters with acute leptospirosis (96).

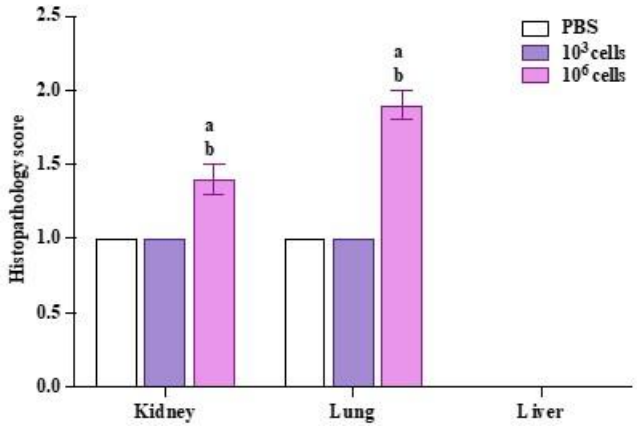

Figure 15 *Histopathology score of kidney, lung, and liver tissues from infected C3H/HeNJ mice.*

C3H/HeNJ mice were infected with either 10^3 cells or 10^6 cells of leptospires, and PBS as a control. The tissues were stained with H&E and the scoring of microscopic lesions was determined by a pathologist with a blinded manner. Each bar represents mean pathology score ± standard deviation (SD). Statistical values were analyzed by Bonfferoni's multiple comparisons test. Letter a and b indicate significant differences compared to the PBS- treated group and the group infected with 10^3 cells of leptospires, respectively.

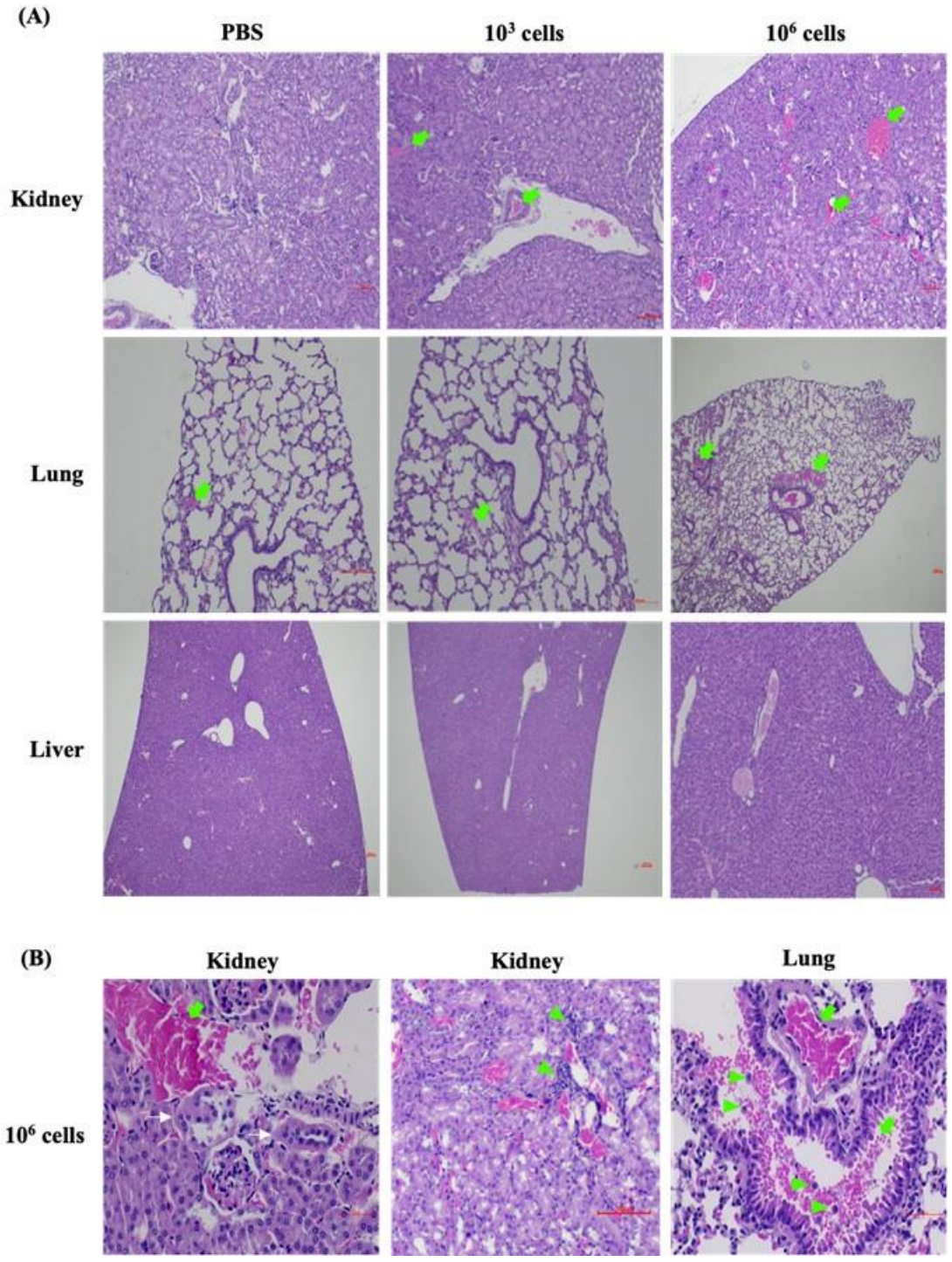

Figure 16 *Histopathology of kidney, lung, and liver tissues from infected C3H/HeNJ mice.*

C3H/HeNJ mice were infected with either 10^3 cells or 10^6 cells of leptospires, and PBS as a control. (A) Kidney, lung, and liver tissues in each group were stained with H&E.

(B) Kidney tissues of infected mice showing hemorrhage (green arrow), mononuclear cell infiltration (green head arrow), and tubular damage (white arrow) were visualized under light microscopy at a magnification of 400×. Marked infiltration of inflammatory cells (green head arrow) and alveolar spaces filled with blood (green arrow) of the infected lung indicates mild alveolar damage.

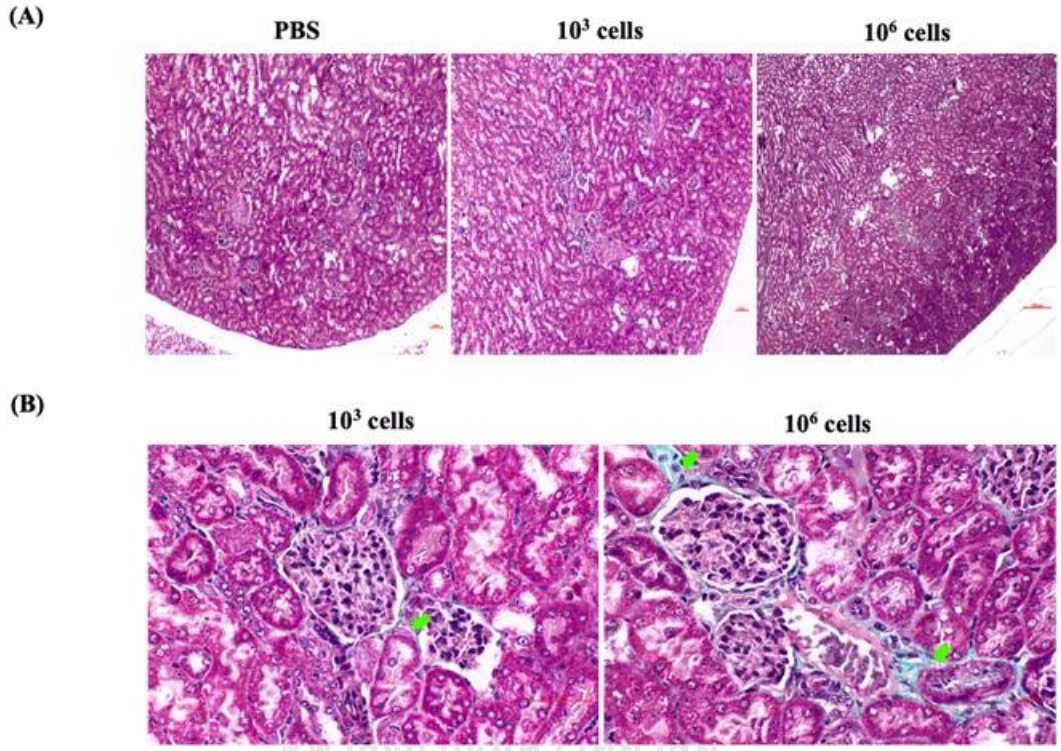

Figure 17 *Detection of fibrosis using Masson trichrome stain of kidney tissues from infected C3H/HeNJ mice.*

C3H/HeNJ mice were infected with either 10^3 cells or 10^6 cells of leptospires, and PBS as a control. (A) Renal collagen deposition in the infected group and PBS-treated group was visualized under light microscopy at a final magnification of 40×. (B) Collagen deposition in the interstitium of the kidney tissues (green arrow) from both infected C3H/HeNJ groups was visualized under light microscopy at a final magnification of 400×.

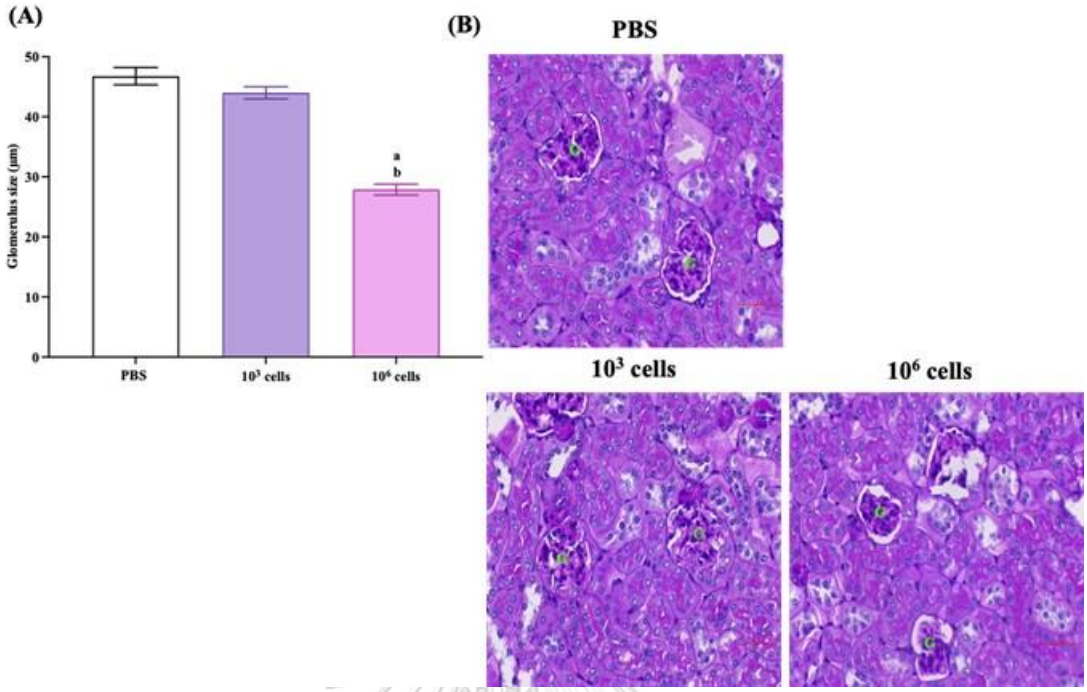

Figure 18 *Measurement of glomerular size using PAS-D stain of kidney tissues from infected*

C3H/HeNJ mice and. C3H/HeNJ mice were infected with either 10^3 cells or 10^6 cells of leptospires, and PBS as a control. (A) Glomerular size of each sample was measured for five fields under light microscopy at a final magnification of 100×. Each bar represents mean glomerular size (µm) ± standard deviation (SD). Statistical values were analyzed by Bonfferoni's multiple comparisons test. Letter a and b indicate significant differences compared to the PBS-treated group and the group infected with 103 cells of leptospires, respectively. (B) Histopathology of the kidney tissues shows shrinkage of glomeruli with reduction of glomerular size in the infected C3H/HeNJ mice. Legend G indicates glomeruli.

Antibody responses against Leptospira in C3H/ HeNJ mice after leptospiral infection

The level of anti-*Leptospira* IgM and IgG in infected mice were measured at 28 days post-infection. At this stage, low IgM levels were detected in sera of all infected mice (Fig. 19A). Total IgG levels of mice infected with 10^6 cells and 10^3 leptospiral cells

were significantly higher than those received PBS. The mice infected with 10^6 cells of leptospires produced significantly higher total IgG levels than those infected with 10^3 cells of leptospires. To determine a predominant IgG istoype, the levels of IgG1 and IgG2a were compared. The levels of IgG2a were higher than IgG1 in all mice infected with *Leptospira*, indicating Th1 responses. However, IgG2a levels were not significantly different from IgG1 in mice infected with 10^3 cells of leptospires (Fig. 19B).

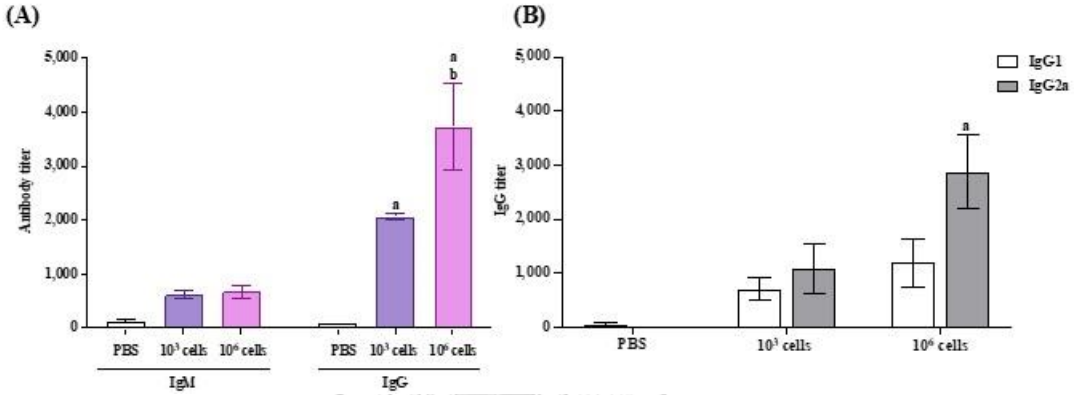

Figure 19 *Humoral immune responses in infected* C3H/HeNJ mice at 28 days post-infection. The antibody titers of (A) IgM and IgG, and (B) IgG1 and IgG2a against whole cell lysates of Leptospira were measured by ELISA. Each bar represents mean antibody titer ± standard deviation (SD). Statistical values were analyzed by Bonfferoni's multiple comparisons test. Letter a and b indicate significant differences compared to the PBS-treated group and the group infected with 103 cells of leptospires, respectively.

IFN-γ and IL-6 production in Leptospira infected C3H/HeNJ mice

The splenocytes of mice infected with 10^6 leptospiral cells produced significantly higher levels of IFN-γ and IL-6, which are proinflammatory cytokines, than mice infected with 10^3 cells of leptospires (Fig. 20A and Fig 20B) and the PBS-treated group. Likewise, the levels of IFN-γ and IL-6 produced by splenocytes of mice infected with 10^3 leptospiral cells were significantly higher than the PBS-treated group. Although the level of IL-10 secreted from splenocytes of mice inoculated with 10^6 and 10^3

leptospiral cells was significantly higher than PBS group, there was no significant different between two groups of mice infected with low and high doses (Fig 20C).

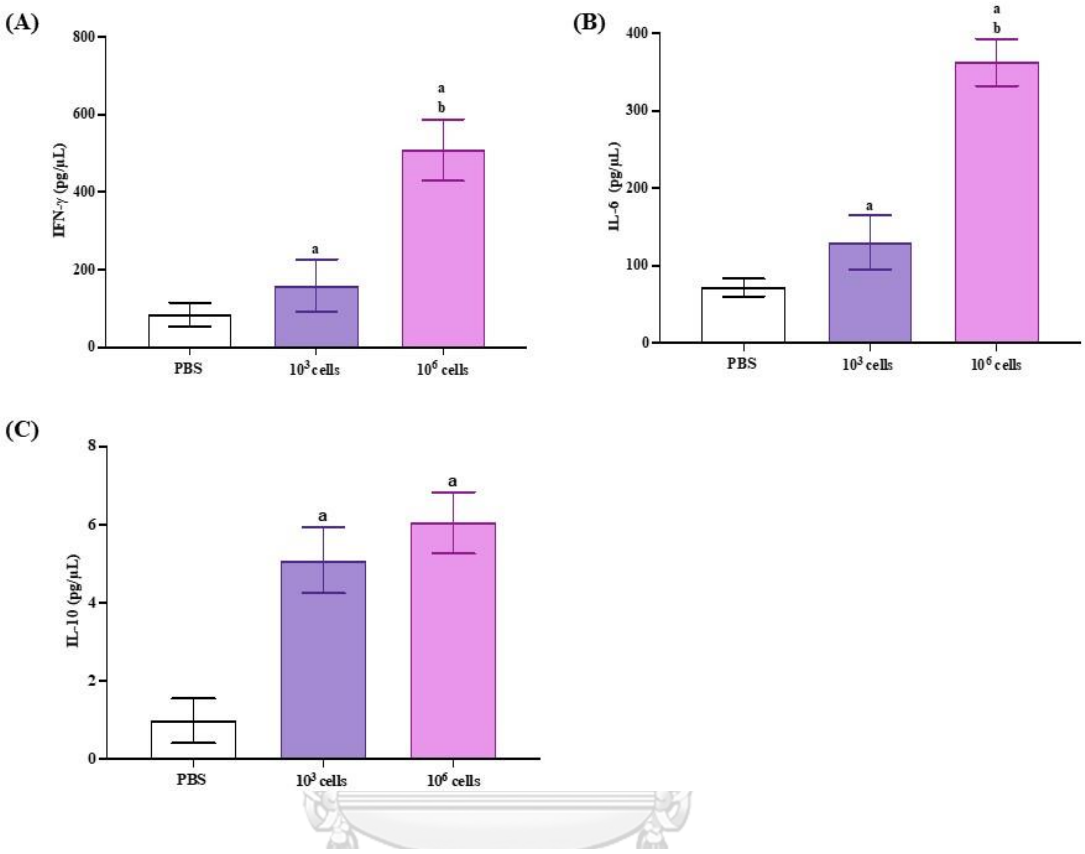

Figure 20 *Cytokine levels produced by splenocytes from infected C3H/HeNJ mice.* The splenocytes from C3H/HeNJ mice infected with either 10^3 cells or 10^6 cells of leptospires, and PBS were incubated with 106 cells/ml of heat-killed L. interrogans serovar Pomona. At 72 h post-stimulation, the cytokine levels of (A) IFN-γ, (B) IL-6, and (C) IL-10 in culture supernatant were measured by ELISA. Each bar represents mean cytokine level ± standard deviation (SD). Statistical values were analyzed by Bonfferoni's multiple comparisons test. Letter a and b indicate significant differences compared to the PBS-treated group and the group infected with 103 cells of leptospires, respectively.

.Discussion

Animal models are essential to investigate the immunopathogenesis of infectious disease including leptospirosis. Hamsters and guinea pigs have previously been used as models of acute leptospirosis (17, 97, 98). However, the limitations on genetic information and biological reagents for both animals hinder the investigation of their immunological changes. On the other hand, mouse models are widely used in immunological research because of comprehensive knowledge of immune response and commercially available immunological reagents. Previous studies on mouse models for lethal, sublethal, and chronic leptospirosis (82, 99) suggested that the immunopathogenesis of leptospirosis depends on strain, genetic background, and age of tested mice as well as infectious dose and serovar of *Leptospira*.

In this study, 5-week-old C3H/HeNJ mice, a crossbreed between C3H/HeN mice (wild-type TLR 4) and C3H/HeJ mice (TLR 4 defective mice), were evaluated for an animal model of leptospirosis. We used virulent low-passage *Leptospira* serovar Pomona that killed hamsters within 2 weeks after infection (Table 1). C3H/HeNJ mice survived up to 28 days after infection with the same dose (10^3) and 1,000 times higher dose (10^6) of leptospires used for hamsters. All infected mice did not present apparent symptoms of leptospirosis. Based on our protocols, C3H/HeNJ mice are not a lethal leptospirosis model. It might be explained by the fact that C3H/HeNJ mice are homozygous for wild-type TLR4 as in C3H/HeN mice (Fig. 13B), which also survived after infection with *L. interrogans* serovar Icterohaemorraghiae (24), suggesting that TLR4 is crucial for controlling leptospiral infection in mice. We confirmed by TLR4 gene sequencing that C3H/HeNJ mice in our study have homozygous wild-type TLR4 (Fig 13B) Although the first-generation offspring (C3H/HeJ) mice should be heterozygous for TLR4, the subsequent offspring of C3H/HeNJ mice should be heterozygous for TLR4, the subsequent offspring of C3H/HeNJ mice might be homozygous wild-type TLr4 after several breeding between heterozygous TLR4 mice as shown in our study. However, the genetic variation or heterozygous loci at other regions affecting phenotype of C3H/HeNJ mice have not been investigated.

Sublethal signs of leptospirosis are characterized by the presence of *Leptospira* in blood, kidney, and urine, as well as weight loss (100, 101). Furthermore, the kidney

pathology shows a typical interstitial nephritis associated with mild renal fibrosis (99, 100, 102, 103). For example, in adult C3H/HeJ mice, the inoculation of 10^8 cells of *L. interrogans* serovar Copenhageni via conjunctival route led to weight loss, leptospiral burden in blood and kidney, renal inflammation, and shedding of *Leptospira* in urine (104). BALB/c mice infected with *L. interrogans* serovar Copenhageni were asymptomatic with renal colonization and urinary shedding of leptospires (105). The infection of leptospires serovar Copenhageni and Manilae in C57BL/6 mice induced interstitial nephritis, renal fibrosis, and urinary *Leptospira* shedding (102). Moreover, tissue fibrosis is a complicated mechanism involving cell differentiation and cell signaling pathways leading to pathologic accretion of extracellular matrix (ECM) components (102, 103, 106). The findings implied that leptospires can destroy epithelial cells with ECM accumulation resulting in fibrosis. In this study, after inoculated with 10^6 leptospiral cells, the C3H/HeNJ mice showed renal colonization of leptospires (Fig. 14), lung and kidney damage (Fig. 15 and Fig. 16), and renal fibrosis (Fig. 17) at 28 days post-infection. Therefore, C3H/HeNJ mice could be used as a sublethal model of leptospirosis.

Antibody-mediated immunity plays a crucial role in host defense against leptospirosis (107), but the major IgG isotype associated with protection has been inconclusive. In sublethal model, the infection of C3H/HeJ mice with *L. interrogans* serovar Copenhageni by eyedrop inoculation mainly induced IgG3 antibody at 2 weeks post-infection (104). However, IgG1 isotype was a predominant antibody in the infected 10- to 14-week-old C3H/HeJ mice after intraperitoneally injected with leptospires serovar Copenhagni (101). In this study, the five-week-old C3H/HeNJ mice intraperitonially inoculated with 10^6 cells of *Leptospira* serovar Pomona significantly produced higher IgG2a than IgG1 antibody (Fig. 19B). In contrast, low-dose inoculum resulted in comparable levels of IgG1 and IgG2a. These evidences implied that IgG class switching in *Leptospira* infection might be affected by various factors, such as route and inoculum dose of infection, strain or serovar of *Leptospira*, age of animals and pattern recognition of Toll-like receptors (108). IgG isotype switching is regulated by T-helper (Th) cells (109, 110). IFN-γ is a major cytokine of Th1 immune response (Moore et al., 2001) which exhibits isotype switching to IgG2a. The infected C3H/HeNJ mice

produced IFN-γ and IgG2a with infectious dose dependent manner (Fig. 19 and Fig 20), indicating Th1-dominated response. In this study, renal colonization of pathogenic leptospires might cause inflammatory response modulated by Th1 response to control leptospiral infection in C3H/HeNJ mice leading to sublethal infection. The role of T cell response in leptospiral pathogenesis requires further investigation.

Leptospires disseminated and colonized renal tubules with inflammatory cytokine activation and fibrosis induction in the surviving C3H/HeNJ mice**.** The infected mice produced IL-6 that plays a crucial role in the recruitment of immune cells and promotes fibrosis (111) in a dose-dependent fashion. Although the infected C3H/HeNJ mice could control and survive *Leptospira* infection, they presented fibrosis in the kidney. These findings suggest that leptospires caused ECM accumulation resulting in renal fibrosis, a histological hallmark of chronic kidney disease (CKD) in C3H/HeNJ mice. However, we did not determine chronic infection of mice as previously performed in the rat model (112).

In summary, five-week-old C3H/HeNJ mice (homozygous wild-type TLR4) infected with *L. interrogans* serovar Pomona survived at least 28 days without clinical signs of leptospirosis. C3H/HeNJ mice infected with 10^6 leptospiral cells presented hemorrhage and moderate mononuclear inflammatory infiltration in the kidney and the lung. Moreover, leptospiral infection caused renal colonization and renal fibrosis. These results indicated that C3H/HeNJ mice might be a new sublethal leptospirosis model. Inoculum dose for each strain of *Leptospira* should be optimized before use. This model may help to better understand the pathogenesis mechanisms of leptospirosis and may be used to test therapeutic and prophylactic strategies for leptospirosis in the future.

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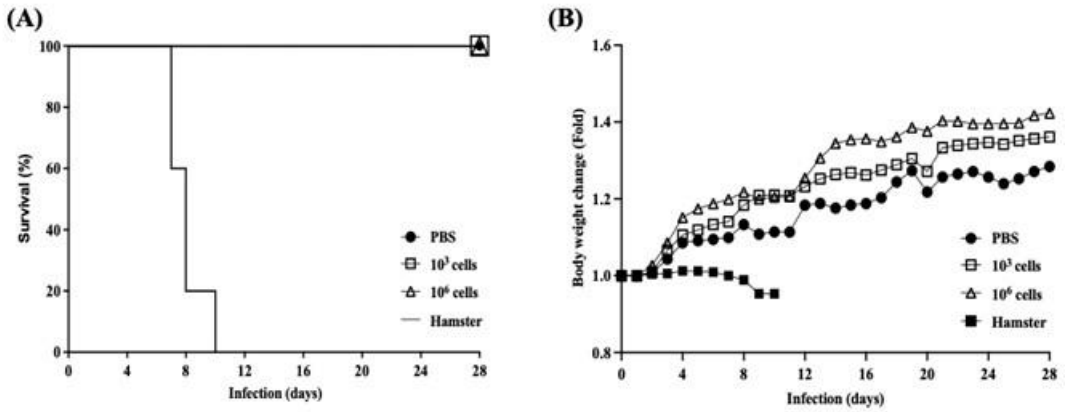
Supplementary

Supplementary Fig. 1. Survival rates and body weight changes of infected C3H/HeNJ mice. (A) Survival rates of the C3H/HeNJ mice infected with either 10^3 cells, 10^6 cells of leptospires, or PBS, and hamsters infected with 10^3 cells of leptospires. (B) Body weight changes of the infected mice and hamsters that were calculated by the percentage of mean body weight (investigated day/first day of infection × 100) compared to 100% of mean body weight on first day of infection (day 0).



SECTION III

Conclusions

Part I:

The responses of human monocyte-derived dendritic cells to pathogenic (serovar Pomona and clinical isolate) and non-pathogenic (serovarPatoc and Ranarum) strains of *Leptospira* spp. were compared. Immature MoDCs showed slight phagocytic acitivity against pathogenic and non-pathogenic strains. However, the pathogenic strains significantly induced more apoptosis, impaired MoDC maturation by CD83 and CD80 reduction, and increased IL-6 production. In case of stimulation with the pathogenic strains, MoDC were suppressed for maturation, naïve $CD4^+$ T cell proliferation was inhibited, and IL-10 production for regulatory T cell activation was induced. In contrast, the non-pathogenic strains stimulated the maturation of MoDCs and induced IL-12p70 and IL-10, leading to an increase in the proliferation of naïve $CD4^+$ T cells and IFN-γ-producing $CD4^+$ for T helper 1 cell activation. In conclusion, the pathogenic strains impaired the function of MoDCs, resulting in no clearance of leptospires. On the contrary, the non-pathogenic strains stimulated MoDC functions to prevent infection.

Part II:

To investigate the susceptibility of C3H/HeNJ mice against *L. interrogans* serovar Pomona. Five-week-old female C3H/HeNJ mice intraperitoneally infected with low ($1x10^3$ cells) and high ($1x10^6$ cells) inoculum doses of *L. interrogans* serovar Pomona did not develop aucte lethal infection and survived up to 28 days post-infection. However, C3H/HeNJ infected with high inoculum dose presented more pathological changes in the lungs and the kidneys including renal fibrosis and glomerular shrinkage that those infected lower dose. Moreover, the level of IFN-γ, IL-6, and IgG2a in the high-dose infected C3H/HeNJ mice were induced at significantly higher levels than those in the low-dose infected mice. Therefore, C3H/HeNJ mice could be used as a sublethal model of leptospirosis.

Limitations

Part I:

1. We used immature MoDCs as an in vitro model to study the difference of DC response to pathogenic and non-pathogenic leptospires. However, MoDC functions might not be exactly extrapolated to cDC.
2. We investigated three cytokines produced by MoDCs that were sufficient to explain the inflammatory responses. However, we can further investigate more cytokines such as TNF-a, IL-2, IL-4, and IL-10, which are related to severe leptospirosis (6, 73), to support the results.
3. We adjusted the P-value from 0.05 to 0.3 in the RNA sequencing analysis to increase the number of DEGs that might be associated with host responses to leptospires. The results should be further confirmed by RT-qPCR.

Part II:

1. We investigated two inoculum doses of leptospires infection. The C3H/HeNJ infected with 10^6 cells of *L. interrogans* serovar Pomona presented a sublethal infection. Therefore, this study should use various inoculum doses and leptospiral strains to obtain more information of the animal model.
2. We determined only a one-time point (day 28 after infection) of the antibody levels of IgM and IgG and cytokine production. This result can elucidate the major types of antibody response and is related to T helper 1 response. In the event of an antibody mechanism, we could investigate the antibody levels and cytokine production at various time points (day 0, 7, 14, and 21) to better understand pathogenesis.
3. We determined that kidney colonization is one characteristic of chronic infection.
4. We should investigate the leptospires shedding in the urine of C3H/HeNJ mice because chronic infection can be strongly confirmed.

Further studies

1. We will find the new candidates of mouse strains for use in lethal models of leptospirosis.
2. We will select genes that might be associated with pathogenesis of leptospiros from the transcriptomics analysis of stimulated MoDCs and validate the genes by RT-qPCR.
3. We will investigate the functions and explore the pathways that might be associated with susceptibility of human hosts to leptospiral infection.

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