ACTIVATION OF DENDRITIC CELLS AND INDUCTION OF CD4(+) T CELL DIFFERENTIATION BY *LEPTOSPIRA INTERROGANS*

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (IMMUNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2007

Thesis

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ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere thank and acknowledge all the people who have helped me to complete this dissertation. I would first to thank my advisor Assoc.Prof Dr. Pattama Ekpo, for teaching me in general knowledges, how to focus the subjects and support along the whole experiments. I would like to express equally thank all my co-advisors Prof. Dr. Kovit Pattanapanyasat, Dr. Sathit Pitchyangkul and Dr. Anneke Engering for their experiences, valuable technique guidance, or tools necessary to complete all steps on the experiments.

My appreciation is also extended to Assoc.Prof Yupin Suputtamongkol at Department of Medicine, Faculty of Medicine Siriraj Hospital, Col.Dr. Duangporn Phulsuksombati and Major Noppadol Sangjun at the Department of Veterinary Medicine, at Royal Thai Army-Armed Forces Research Institute of Medical Sciences (RTA-AFRIMS) and Assoc.Prof Dr. Chintana Chirathaworn for their provision of valuable bacteria using in this study. I also would like to thank Dr. Yvette van Kooyk at Vrije University Medical Center Amsterdam, The Netherlands for soluble DC-SIGN and DC-SIGN transfected cell line.

I also would like to thank Prof. Dr. Stitaya Sirisinha for his valuable comment in the study.

I am grateful that I have an excellent group of people to work with. I greatfully thank all staffs at the Department of Immunology, United States Army Medical Component-Armed Forces Research Institute of Medical Sciences (USAMC-AFRIMS), Mr. Kosol Yongvanitchit at for his expert experience in flow cytometry that very helpful to correct the results, Mrs. Amporn Limsalakpetch and Mrs Utaiwan Kum-Arb for their friendship, assistance and always willing to help. I would like to thank Mr. Utane Rungpanich and Ms Nujorn Chansong at Department of Immunology, Faculty of Medicine Siriraj Hospital for bacteria culturing technique and other supports.

I also would like to special thank for all blood donors for volunteering their valuable bloods along this study.

I would like to thank Siriraj Graduate Thesis Scholarship from the Faculty of Medicine Siriraj Hospital, Mahidol University for partly financial supported which enabled me to undertaken this study.

I thank all of friends, staffs and graduate students at Faculty of Medicine Siriraj, Mahidol University for their contribution and made this study more enjoyable. Finally, great appreciation is especially expressed to my family, Mr Amarin and Mrs Duanchai Kohman and Dr. Puapong Kongsaeree for their love, helps, support, inspiration and cheering me up.

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ACTIVATION OF DENDRITIC CELLS AND INDUCTION OF CD4(+) T CELL DIFFERENTIATION BY *LEPTOSPIRA INTERROGANS*.

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ABSTRACT

Worldwide leptospirosis is the most widely occuring zoonosis disease caused by *L. interrogans*. It is an emerging problem causing public health and also livestock problems. Pathogenesis, immune responses and cellular receptors for leptospiral are not well understood. DC-SIGN is one of the most extensive PRRs that expresses on DCs that recognizes carbohydrate structures. Reference strains of *L. interrogans* and *L. biflexa* serovar Patoc I were used for screening the carbohydrate on the leptospiral surfaces. The common backbone structure of pathogenic *L. interrogans* species contains mostly mannan as a major carbohydrate component similar to nonpathogenic *L. biflexa* serovar Patoc I. Four representative serovars common in Thailand were used as *L. interrogans* serovar Pyrogenes 2317 both virulent and avirulent strains and serovar Autumnalis L-643 and BL-6 strains which were isolated from deceased and recovered leptospirosis patients, respectively. All tested leptospires were recognized with soluble chimeric DC-SIGN Fc which has been used in ELISA to screen for specific carbohydrates that are recognized by DC-SIGN. DC-SIGN expressing cells as K-562 transfected DC-SIGN, K-SIGN, and MoDCs were able to specifically recognize *L. interrogans* since competing ligands such as mannan, EDTA and anti-DC-SIGN antibody reduced the binding on these cells. *L. interrogans* stimulated DCs maturation as up-regulation of costimulatory molecules, CD83 and CD86. However, there were strikingly different effects on IL-10, IL-12p70 and TNF-α productions. Only high amounts of IL-12p70 in the supernatants of DCs stimulated with *L. interrogans* serovar Pyrogenes 2317 strains were confirmed with the IFN-γ production by T cells though the virulent strain that showed higher levels. *L. interrogans* activated naïve T cell response in non specific polarization as mixed Th1/Th2. The reduction in IL-12p70 and TNF-α might be involved in immunopathogenesis and mixed Th1/Th2 responses that are crucial to virulence. Both the virulence properties of *L. interrogans* strains and host factors may strongly influence the clinical outcome of the infection. Our data demonstrated that *L. interrogans* bind DC-SIGN, induced DCs maturation and IL-12p70 and TNF-α production and trigger naïve T cell stimulation. This finding has increased knowledge of leptospiral cell receptors and interaction on DCs and T cells stimulation that have important implications for future vaccine development and immunotherapy.

KEY WORDS: *L. interrogans* / DC-SIGN / Dendritic cells/ naïve T cells
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การกระตุ้นเดนไดร์ติกเซลล์และการเหนี่ยวนำการเปลี่ยนแปลงของ CD4(+) T cells โดยจุลชีพเลปโตสไปร่า

(ACTIVATION OF DENDRITIC CELLS AND INDUCTION OF CD4(+) T CELL DIFFERENTIATION BY *LEPTOSPIRA INTERROGANS)*

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บทคัดย่อ

โรคเลปโตสไปโรซิส เป็นโรคติดเชื้อที่ติดต่อจากสัตว์สู่คนมีสาเหตุมาจากจุลชีพก่อโรคลักษณะเกลียวที่เรียกว่าเลปโตสไปร่า โดยเฉพาะกลุ่ม *L. interrogans* โรคนี้พบกระจายอยู่ทั่วโลกโดยพบมากในประเทศเขตร้อน ในประเทศไทยพบผู้ป่วยโรคเลปโตสไปโรซิสทั่วประเทศและพบมากทางภาคเหนือและภาคตะวันออก เฉียงเหนือ อาการของโรคมีทั้งที่ไม่ปรากฏอาการจนถึงมีอาการรุนแรงถึงขั้นเสียชีวิต เพื่อเพิ่มความเข้าใจลักษณะทางพยาธิวิทยาของการก่อโรคเลปโตสไปโรซิส ในการเข้าสู่เซลล์ และการกระตุ้นให้ระบบภูมิคุ้มกันตอบสนอง การศึกษานี้จึงได้ทำการศึกษาปฏิสัมพันธ์ระหว่างเชื้อเลปโตสไปร่าและเดนไดร์ติกเซลล์ (DCs) และการกระตุ้นการหลั่งไซโตไคน์ของทีเซลล์ (T cells) จากการศึกษาพบว่าเชื้อเลโตสไปร่า 23 serovars พบว่ามีลักษณะโครงสร้างหลักบนผิวเซลล์ประกอบด้วยน้ำตาลแมนโนส ซึ่งระบบภูมิคุ้มกันแบบไม่จำเพาะ มีตัวรับที่มีความจำเพาะต่อน้ำตาลแมนโนสอยู่หลายชนิด ชนิดที่ได้รับความสนใจมากคือชนิดหนึ่งคือ DC-SIGN ที่อยู่บนผิวของเดนไดร์ติกเซลล์ เมื่อทดลองโดยใช้ *L. interrogans* serovar Pyrognes 2137 สายพันธุ์ทั้งที่มีความรุนแรงและที่ทำให้อ่อนแรง รวมทั้งสายพันธุ์ Autumnalis L-643 และ BL-6 ที่แยกได้จากผู้ป่วยที่เสียชีวิต และ ผู้ป่วยที่หายจากโรคเลปโตสไปโรซิส มาจับกับตัวรับ DC-SIGN ทั้งแบบที่เชื่อมต่อกับอิมมูโนโกลบูลินจี (soluble-DC-SIGN-Fc-IgG) และบน K-562 เซลล์ที่มีการเติมยีนส์ของ DC-SIGN (K-SIGN) และเดนไดร์ติดเซลล์ปกติ พบว่าเชื้อเลปโตสไปร่ามีการจับกับกับ DC-SIGN อย่างจำเพาะ นอกจากนี้ยังสามารถกระตุ้นให้เดนไดร์ติกเซลล์เกิดภาวะ maturation โดยมีการเพิ่มขึ้นของ molecule CD83 และ CD86 และหลั่งสารไซโตไคน์ IL-10 และ IL12p70 และ TNF-α ได้ แต่แตกต่างกันไปในแต่ละสายพันธ์ เมื่อทดสอบดูความสามารถในการกระตุ้นเซลล์ชนิด naïve T cells พบว่าเชื้อเลปโตสไปร่าทั้งสอง สายพันธุ์สามารถกระตุ้นให้เกิดการสร้างทีเซลล์ทั้ง Th1 และ Th2 เซลล์ซึ่งดูได้จากการสร้างสารไซโตไคน์ภายในเซลล์ IFN-γ และ IL-4 ตามลำดับ ทั้งนี้ความแตกต่างที่เกิดขึ้นอาจเนื่องจากปัจจัยจากเชื้อเลปโตสไปร่าเองและปัจจัยจากผู้ป่วยและเซลล์ที่เกี่ยวข้อง

การศึกษานี้ได้เพิ่มเติมความรู้ในเรื่องตัวรับ DC-SIGN สามารถจับอย่างจำเพาะกับเชื้อเลปโตสไปร่า และกระตุ้นให้เดนไดร์ติกเซลล์เกิดภาวะ maturation และหลั่งสารไซโตไคน์ IL-10, IL12p70 และ TNF-α ซึ่งมีผลให้ทีเซลล์เกิดการตอบสนองทั้งแบบ Th1 และ Th2

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LIST OF ABBREVATION

Abbreviations	Term
APCs	Antigen-presenting cells
ARF	Acute renal failure
CAAT	Cross agglutination absorption test
CLRs	C-type lectins
ConA	Concanavalin A
CRD	Carbohydrate-recognition domain
CSF	Cerebrospinal fluid
DBA	*Dolichos biflorus* agglutinin
DC	Dendritic cell
DC-SIGN	Dendritic cell specific ICAM-3 grabbing non-integrin
DNPP	Diethanolamine/*p*-nitrophenyl phosphate
ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinghausen McCullough-Johnson-Harris
FBS	Fetal bovine serum
FCS	Fetal calf serum
Fuc	Fucose
Gal	**G**alactose
GalNAc	*N*-acetylgalactosamine
GLPs	Glycolipoproteins
h	Hour
Hap1	Hemolysis-associated protein1
Hsp15	Heat shock protein 15
K-SIGN	K-562 DC-SIGN transfected cells
Lig	Leptospiral Immunoglobulin-like
LPS	Lipopolysaccharide
Man	Mannose
MAT	Microscopic agglutination test

LIST OF ABBREVATION (Continued)

Abbreviations	Term
MDCK	Madin-Darby canine kidney cell
MFI	Mean fluorescence intensity
Mg	Milligram
μg	Microgram
MMR	Macrophage mannose receptor
MoDCs	Monocytes derived dendritic cells
MOP	Ministry of Public Health
NIAH	National Institute of Animal Health
NIH	National Institute of Health
OMPs	Outer membrane proteins
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PNA	Peanut agglutinin lectin
PRRs	Pattern recognition receptors
RCA_{120}	*Ricinus communis* agglutinin
SBA	Soybean agglutinin
SphA	Sphingomyelinase C
SphH	Sphingomyelinase H
SPF	specific pathogen free
SRBC	Sheep red blood cells
ST-AP	Streptavidin-alkaline phosphatase
TLRs	Toll like receptors
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells
TSM	Tris-saline magnesium buffer
TSM/T	TSM containing 0.05% Tween 20
UEA1	*Ulex europaeus* agglutinin
WGA	Wheat germ agglutinin

CHAPTER I
INTRODUCTION

Worldwide leptospirosis is the most widely occuring zoonosis disease with a much greater incidence in tropical regions such as Nicaragua, Brazil, India, southeast Asia and the United States also [1]. Leptospirosis is also cause the problem for human and livestock in Thailand. Leptospirosis is transmitted either by direct contact with infected animals or by exposure to water or soil contaminated by the urine of infected animals. The epidemiology of leptospirosis involved in changes in animal husbandry, climate, and human activities. The disease is caused by infection with pathogenic *Leptospira* species, especially *L. interrogans*. The clinical symptoms are extremely wide, ranging from subclinical infection to a severe syndrome of multiorgan infection with high mortality.

The strategies to counter leptospiral infection requires information on cellular interaction and mechanisms of infection. There are limited knowledge available for these topics. Interactions between leptospires and eukaryotic cells have been studied in many aspects. Examples are phagocytosis, adhesion molecules on target cells, specific antibody detection and cellular immune responses. Adherence of leptospires to host mammalian cells plays an essential role in the pathogenesis of leptospirosis since leptospires migrate through the bloodstream to target tissues, colonize then cause pathogenesis in host. Moreover, there were some reports showing that *Leptospira* induced apoptosis in macrophages and hepatocytes [2, 3]. In general, *Leptospira* enters human body through skin and killed by phagocytic cells such as monocytes, monocyte-derived macrophages and neutrophils of innate immune system. However, DCs which are the professional antigen presenting cells lining at this site may be important for introduce immune response against leptospira.

DCs express numerous highly conserved pattern recognition receptors (PRRs) and the one important receptor is DC-SIGN, DC-Specific ICAM-3-Grabbing Non-

integrin or CD209 [4]. This receptor plays role in both pathogen recognition and cell migration. DC-SIGN recognizes high-mannose glycans as well as fucose-containing Lewis antigens present on many different pathogens such as viruses, bacteria, fungi and parasite [5-10].

After DCs capturing antigens, they attract T-cells, present their antigen load, activate the T-cells and initiate the immune response. Moreover, DCs also play an important role in selecting the type of immune response by polarizing naïve T cells toward either Th1, Th2 or regulatory T cells. The cytokines produced by DCs are keys in determining the type of T-cell response generated for the determination of differentiation of Th cells toward Th1 and Th2 cells that regulate cellular and humoral immune response. There is no report of study on interacting *Leptospira* with DCs. Based on DC-SIGN carbohydrate recognition profile, chemical and structural compositions of *Leptospira*, DC-SIGN might function as receptor for *Leptospira* in innate immunity. Soluble DC-SIGN-Fc as well as cell line transfected with DC-SIGN and monocytes-derived DCs showed DC-SIGN specifically recognize *Leptospira* as result of common backbone carbohydrates on their surface. This recognition integrates adaptive immune response via DCs by induce DCs maturation and initiate naïve T cells activation to produce Th1 and Th2 cytokines.

CHAPTER II
OBJECTIVES

Innate immune system provides a first line of host defense and is essential for the control of bacterial infection and subsequent dictate adaptive immune response. Leptospira infection mainly via skin that DCs are lining. DCs express DC-SIGN receptor that recognize specific carbohydrates containing high mannose and fucose molecules which present on the surface of pathogens. By chemical and structural compositions, *Leptospira* contains LPS at the surface in high percentage of carbohydrates. These molecules may play some roles in immune activation and integrate adaptive immune response via DCs. Further more, when DCs initiate naïve T cell activation, these cells also play important roles in Th1/2 polarization and activate different immune response toward either cellular or humoral immune response. Then, it is interesting to study the interaction between *Leptospira* and DCs via DC-SIGN. This study may give some information for better understanding the immune response against leptospirosis in term of receptor usage, cytokine productions and immune responses on DCs.

The objectives of this study are to:

1. Investigate the interaction of DC-SIGN with pathogenic *Leptospira*.
2. Determine whether the monocyte-derived DC response to *Leptospira*.
3. Determine the cytokine-based immune polarization of the interactions between *Leptospira* and DC whether Th1 or Th2 cytokines polarization.

CHAPTER III
LITERATURE REVIEWS

1. Bacteriology

1.1 *Leptospira* Characteristics

The leptospires are spirochetes that belong to the family *Leptospiraceae* in the order *Spirochaetales.* Leptospires are highly motile, obligate aerobic spirochete that share features of both Gram-positive and Gram-negative bacteria. *Leptospira* has been classified by two categories as serological typing and genotyping.

Serological typing : *Leptospira* was divided by serological classification methods into two species, *L. interrogans* and *L. biflexa*, that containing pathogenic strains and nonpathogenic strains, respectively. Lipopolysaccharide (LPS) is the major antigen involved in serological classification that divided both *L. interrogans* and *L. biflexa* into numerous serovars. The serological typing subdivided *L. interrogans* into over 250 serovars according to the microscopic agglutination test and cross agglutination absorption test (CAAT) [11]. Antigenic related serovars have been grouped into serogroups that have no taxonomic standing but useful for epidemiological understanding.

Genotyping : The genotypic classification based on DNA hybridization divides *Leptospira* into 17 species [12, 13]. The genomospecies of *Leptospira* do not correspond to the serological pathogenic and nonpathogenic serovars (*L. interrogans* and *L. biflexa*) since both serovars occur within the same species. Thus, neither serogroup nor serovar reliably predicts the species of *Leptospira.* This molecular classification is problematic for the clinical microbiologist, because it is clearly incompatible with the system of serogroups.

1.2 Biology of Leptospira

Leptospires are tightly coiled spirochetes (Figure 1). The helical amplitude is approximately 0.1 to 0.15 µm, and the wavelength is approximately 0.5 µm. Although *Leptospira* has general characteristic like other Gram negative bacteria, it has a unique structure of flagella. Two axial filaments which called endoflagellum with polar insertions are located in the periplasmic space and have pointed ends, either one or both which are usually bent into a distinctive hook. This characteristic causes rapid movement of *Leptospira.*

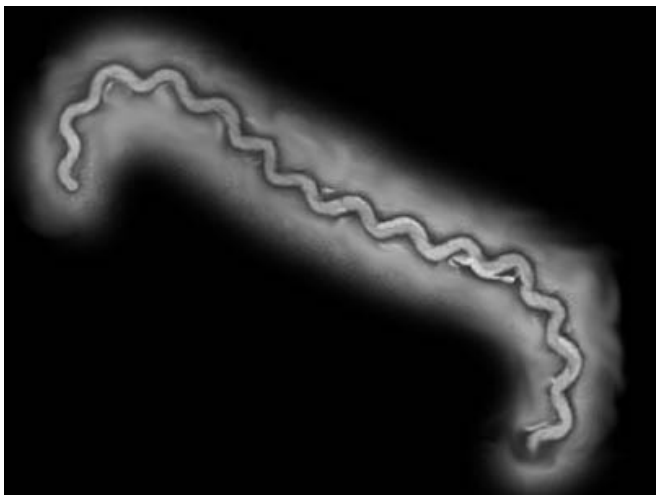

Figure 1 *Leptospira* unique morphology scanning electron micrograph (http://ww2.mcgill.ca/Redpath/Leptospira.jpg)

Pathogenic leptospires survive in moist soil and fresh water for long period of time, especially when the pH is slightly alkaline. These are results of higher viscosity and that favour the cell aggregation, facilitates spirochetes translational motility and chemotaxis and high salts could be inhibitory for pathogenic *Leptospira* under starvation [14, 15].

Leptospira composed of lipopolysaccharide (LPS), proteins and lipids. Leptospiral LPS has a composition similar to that of other Gram-negative bacteria, but has lower endotoxic activity [11]. *Leptospira* contains several proteins and they are involved in virulence and survival in host or adaptive in environments [16-18]. They also contain high lipid content which is about 14-26% of dry weight, and also unusual fatty acids [11]. However, leptospiral lipids did not active biologically since they did

not stimulate the production of antibodies or confer immunity in mice, rabbit or hamster though they can fixed complement [11].

1.3 Molecular Biology

Leptospires are phylogenetically related to other spirochetes [19]. The *L. interrogans* genome is approximately 4,700 kb in size which much higher than other spirochetes. The genome is comprised of two circular chromosomes, a large 4,332 kb chromosome and a smaller 358 kb chromosome [20]. Leptospires contain two sets of 16S and 23S rRNA genes but only one 5S rRNA gene, and the rRNA genes are widely spaced [21]. Leptospiral genomes and genes have been cloned and analyzed [22-24]. Though pathogenic *Leptospira* species share a common evolution that distinct from nonpathogenic species, these genetic differentiation between pathogenic and nonpathogenic serovars were identified. Some of these genes might be responsible for virulence, and transmission capacity [25]. *Leptospira* rearrangements of large segments of chromosome that might cause heterogeneity and variable within species [22]. Structural heterogeneity in the carbohydrate component of LPS moieties derived from difference genes involved in LPS biosynthesis appears to be the basis for the large degree of antigenic variation observed among serovars [23].

1.4 Leptospirosis

Leptospiral transmission involves either direct or indirect exposure to organisms. Direct transmission occurs via blood or shedding urine of infected animals while indirect transmission mostly via contaminated environments such as soil, mud and water [14]. The most common portal of entry is through skin by abrasions or after prolonged immersion in water or via the conjunctiva [11]. After infection, pathogenic leptospires invade into blood circulation and spread rapidly into the target organs which are affecting throughout the body.

Leptospiral genetic diversity reflects the broad ranges of maintenance host species that also impact on human health and livestock production. Leptospires infect several animals and vary from area to area. The small mammals such as rat, dogs, cattle and pigs, are the most common maintenance hosts which may transfer the bacteria to human. Rats and other rodents recognized as the most important reservoirs for leptospirosis [26, 27]. The prevalence of different leptospiral serovars within

human population depends on the reservoir animals and their carried serovars as well as environmental conditions, occupation and agricultural practices [14].

The maintenance species rarely exhibit clinical signs of disease whereas infection of incidence hosts often results in either anicteric or icteric leptospirosis. Anicteric leptospirosis is the majority of infection caused either subclinical or very mild severity. This anicteric syndrome usually lasts for about a week, and its resolution coincides with the appearance of antibodies. Icteric leptospirosis is a much more severe enough to cause death (Weil's Disease). Icteric form range between 5 to 10% of all patients. The complications of severe leptospirosis emphasize the multisystemic nature of the disease. Patients are suffer from a very rapidly progressive severe disease lead to culminating in organs failure such as acute renal failure (ARF) and pulmonary hemorrhage [14]. Uveitis is a potentially chronic condition that may present weeks, months, or occasionally years that found in leptospirosis of human and horses which can develop early or late in disease [28, 29].

The clinical presentation of leptospirosis is biphasic (Figure 2), with the acute or septicemia phase lasting about a week, followed by the immune phase. The incubation period is usually 5 to 14 days, range two to 30 days [11].

1.5 Leptospirosis Epidemic

Leptospirosis is seasonal, with peak incidence in rainy season or after flood and also emerged as a disease of the adventure travelers, especially affecting participants in water-sports [1, 30]. It has a worldwide distribution but significantly higher in the tropical area where transmission conditions are particularly favorable. Currently, the official website of the International Society for Infectious Diseases (www.promedmail.org) reported that leptospirosis marked increased number of cases and frequent outbreaks from disaster of the Asian tsunami of December 2004 and New Orleans flood of August 2005.

Leptospirosis was first reported in Thailand in 1942. Data reported from Ministry of Public Health (MOP) Thailand, indicated a dramatically increase in leptospirosis cases. The disease was seen in all age groups except in children younger than 5 years of age and the peak was in middle age group, 25 to 45 years old, accounting for 80% of all cases.

The highest number of reported cases in Thailand that have been reported was in year 2000 as 23.13 per 100,000 population reported by MOP, Thailand. In 2006, leptospirosis cases was declined and estimated annual incidence was 6.13 per 100,000 population. The most cases throughout this period were reported in north and northeastern [31]. Pulmonary haemorrhage was the major cause of death [32]. In the late of 1990s, seroprevalence survey found that Bratislava, Autumnalis, Pyrogenes and Sejroe are the most endermic serovars**.** Recently, the endermic serovars were changed. The most prevalence serovars isolated was *L. interrogans* serovar Autumnalis [31]. The cause and trigger factors of the epidemic are still unknown. The possible reason might be due to climatologically and ecological conditions that favor the transmission of disease during rainy season, the changing ecology and epidemiology of domestic animals and changes in agricultural practices, increase in rodent density resulted in an increase risk of infection, shift in the predominant infected serovars.

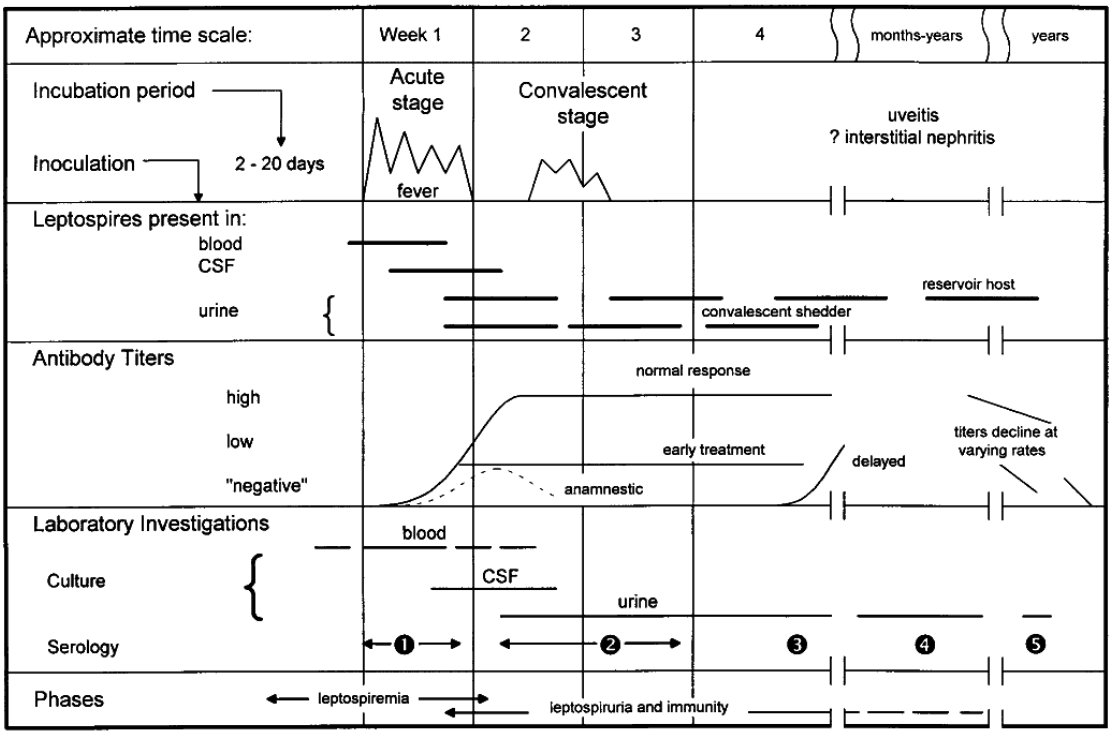

Figure 2 Biphasic nature of leptospirosis and relevant investigations at different stages of disease [14] .

2. Pathology and Pathogenesis

The mechanisms by which leptospires cause disease have only been partially elucidated. The histopathology of leptospirosis is most marked in the liver, kidneys, heart, and lungs, but other organs may also be affected according to the severity of the individual infection [33-36]. Leptospires can be seen within the endothelial cells and renal tubules [35]. Motility is probably important in initial infection and in dissemination of organisms from the site of entry to sites of end-organ damage [37]. However, currently the mechanisms by which leptospires cause disease are still poorly understood. Leptospirosis presumably relates to the inoculums size during infection, host factors and the pathogen's virulence [11]. Adhesion and penetration epithelial and endothelial cells may account for their survival and rapid spread from blood stream to tissues of the pathogenic isolates. The damage of the affected tissues is the result of both an immunopathological process of the host's immune system and the leptospiral itself that greatly affects the outcome of the infection [11].

2.1 Virulence Factors

Leptospires have a typical double membrane structure in common with other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are associated and covered by an outer membrane as shows in Figure 3 [37]. *Leptospira* has a range of potential cellular components that may participate in the pathogenesis such as LPS, glycolipoprotein, peptidoglycan, flagellin and others. The outer membrane of leptospires also contains lipoproteins (outer membrane proteins) and glycoproteins. These molecules are facilitating leptospiral infection by direct contact to the host cells during infection, attachment, penetration, induce immune response or immunopathology and tissue damage.

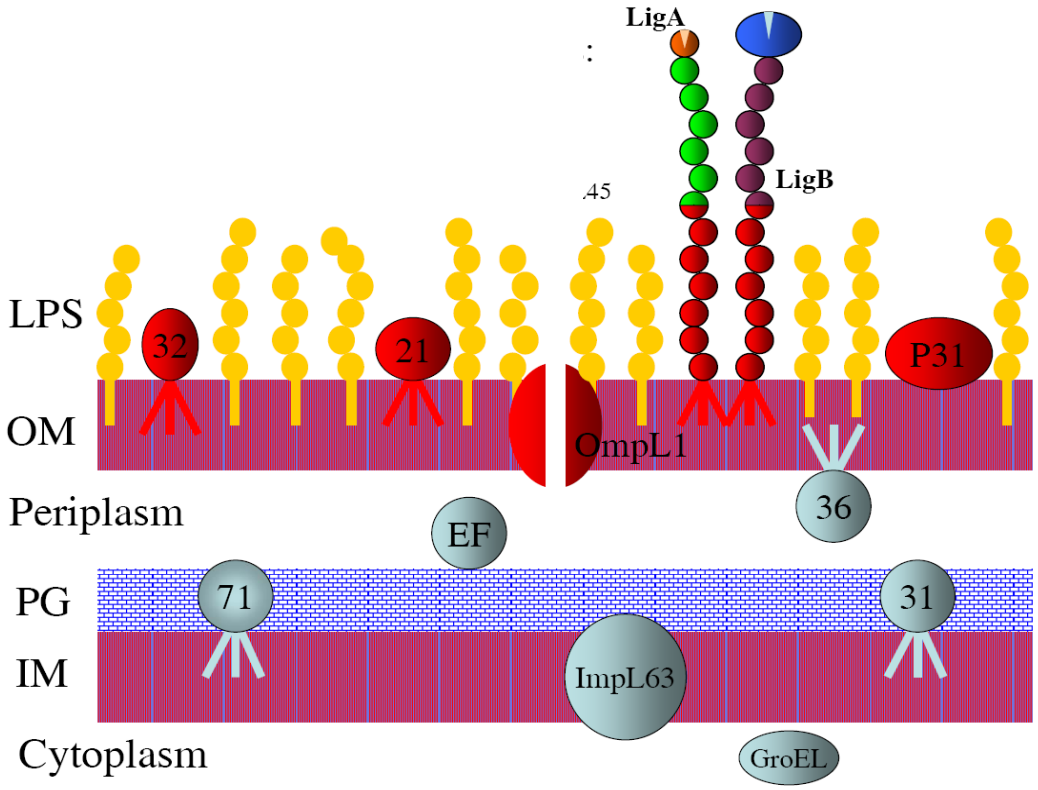

Figure 3 Model of leptospiral membrane architecture applied from Nascimento *et al.* [37]. *Leptospira* have two membranes, an outer and inner membrane. The peptidoglycan cell wall is closely associated with the inner side. The surface is dominated by LPS.

2.1.1 Lipopolysaccharide (LPS)

Leptospiral LPS is the main surface which high immunogenic and responses for serovar specificity. Leptospiral LPS biochemical, physical, and biological properties differ from typical Gram negative bacterial LPS and considered less toxic [11]. The epitopes for serovar specificity are small oligosaccharides derived from the polysaccharides of LPS. The chemical composition of the polysaccharide component of LPS has been analyzed in few strains. The preparation of LPS from *L. interrogans* serovars Copenhagini contains 51% carbohydrates but the proportion of total carbohydrate content and their distribution varies between serovars. The sugar found in leptospiral LPS are arabinose, xylose, rhamnose, fucose, ribose, glucose, mannose, galactose, galactosamine, glucosamine, mannosamine, glucose-6-phosphate and N-acetyl glucosamine [38, 39]. The leptospiral LPS induces apoptosis of lymphocytes via induction of tumor necrosis factor alpha (TNF-α) [40]. The elevated levels of inflammatory cytokines such as TNF-α have been reported in patients with severe leptospirosis [39, 41].

2.1.2 Outer membrane proteins (OMPs)

The outer membrane proteins (OMPs) are position to interact with host cell and contains several virulent factors. These proteins are conserved among pathogenic serovars. Global expression analysis of the *L. interrogans* outer membrane proteome also revealed changes in expression in responses to simulation *in vivo* [42]. OMPs in pathogenic leptospires may be responsible for renal tubular injury and inflammation through NF-κB associated gene expression since the transcription factor NF-κB, further induced increased expression of iNOS, MCP-1 [35, 43, 44]. These responses will enhance cellular injury, cell recruitment and inflammatory process leading to tubulo-intestinal nephritis. These OMPs are differ in their functions. OmpL1 is a transmembrane outer membrane protein function as a heat-modifiable porin [45, 46]. Omp52 surface–exposed membrane protein which regulate growth-phase and may play roles in the interaction of host cells and pathogen during infection since it was detected in patient's sera [47].

2.1.2.1 Lipoproteins

The most abundant proteins in spirochetes are lipoproteins which are also potential toxic factors in leptospirosis through their ability to trigger the host inflammatory response and can induce antibody in infected persons and animals. Several lipoproteins such as LipL21, LipL32, LipL36, LipL41, LipL45 and LipL48 are presented in pathogenic but not nonpathogenic strain and involved in the infectious process in *in vitro* culture [48-51]. However, they are differentially expressed, presumably for the purpose of adapting to different environmental conditions and potentially significant role in the host-pathogen interaction during infection. For example, LipL32 and LipL41 appear to be expressed constitutively by all pathogenic *Leptospira* species under all environmental conditions, while LipL36 and a LipL48, are not expressed during infection, but are expressed in large amounts in culture-attenuated organisms [18, 52, 53]. LipL45 is expressed in early-passage cultures isolated from hamsters infected with *L. kirschneri*, but not in high-passage cultures of the same strain. LipL45 is processed to a 31-kD growth phase regulated peripheral membrane protein, designed $P31_{LipL45}$, which is expressed in both low and high passage cultures [54]. Although several *Leptospira* virulence factors may contribute to the pathogenesis, their pathogenic mechanisms have not been clearly understood.

The most abundant proteins in *Leptospira* is LipL32 or Hap1 (Hemolysis-associated protein1) which can induce high level of anti-LipL32 in serum of leptospirosis patient [49, 50, 55]. Moreover, the recombinant Hap1 showed cross-protective effect with pathogenic strains of *Leptospira* in gerbils [56]. It is a hemolysin secreted protein which is less potent than sphingomyelinase H (SphH) [57]. LipL41 is a surface–exposed lipoprotein that provides synergistic immunoprotection with OmpL1 in the hamster model [58].

The other proteins believed to play role in pathogenesis are Qlp42 lipoprotein, heat shock protein 15 (Hsp15), LruA, LruB and LfhA proteins [59-61]. Qlp42 and Hsp15 are thermoinduced proteins that might play role in the adaptive response of pathogenic leptospires to higher temperatures when they are encountering during infection [59]. LruA and LruB are the inner membrane proteins that restricted to pathogenic leptospires and associated with the peptidoglycan binding

and iron metabolism, respectively. They are detected in horse uveitis and involved significantly high level of IgG and IgA during secondary immune response. They may be involved the early phase of immunopathogenesis since immune privilege is permissive of intraocular immune response that are not proinflammation [60]. However, the pathogenesis mechanism caused by LruA and LruB is unknown.

LfhA is presented in both outer membrane and periplasmic fraction. LfhA specifically interacts with a site within factor H. LfhA is expressed during natural infection and appears to be highly antigenic. It is possible that antibodies which bind to LfhA may also physically prevent factor H binding thereby increasing the susceptibility of the bacteria to complement–mediated killing. Attachment of factor H to surface of *Leptospira* by the conserved LfhA may inactivate C3b and interfere with the antibacterial action of complement [61].

2.1.2.2 Glycolipoprotein and Peptidoglycan

Leptospiral glycolipoproteins (GLPs) can be detected in the organisms recovered at the late phase of disease as granules both in cytoplasm and on the cell membrane in the damaged tissues adhering to endothelial cells, epithelial membranes and macrophages [62-64]. These GLPs display endotoxic properties by inhibiting the tubular epithelial cell sodium pump or Na^+, K^+-ATPase activity in the kidney [65, 66]. The cytotoxic component of GLP was found in the lipid moiety. The inhibitory activity was associated with unsaturated fatty acids, particularly palmitic and oleic acids [67]. The toxic effects can be found in culture cells that lead to cell membrane leakage and cell death [63]. Its presence might be due to the result of partial leptospiral degradation secondary to phagocytosis. Leptospiral peptidoglycan has been reported that it can induce of TNF-α production by monocytes and directly activate cultured human endothelial cell to increase their adhesiveness for neutrophils [68, 69].

2.1.3 Host cell membrane degradation

Leptospira contains proteins that induce cytolysis and play an important role in the virulence and pathogenesis. These proteins including hemolysin, sphingomyelinase, and phospholipase are only produced by pathogenic leptospires [11, 57]. Sphingomyelinase C (SphA) shows sphingomyelinase activity whereas SphH which is a hemolysin does not have sphingomyelinase or phospholipase activities but it acts as pore-forming protein and it is highly conserved among pathogenic leptospires [57]. Phospholipase C acts on erythrocyte and other cell membranes containing phospholipids, resulting in cytolysis.

In addition, number of genes were identified as protease encoding gene including a collagenase, metalloprotease, and several thermolysin orthologs. These enzymes conduct proteolysis proteins that interfere the catalytic metabolism, abolish protein function, destroy extracellular structure and cause cell damage.

2.1.4 Attachment and colonization factors

To initiate infection, *Leptospira* must adhere to tissues in order not to be removed by the physical defenses of host. Once adhered, *Leptospira* colonize to the host tissues such as fibroblasts, renal epithelial cells, or human endothelial cells [2, 70-72]. *Leptospira* has a range of potential virulence factors which may facilitate attachment and colonization such as unique morphology of endoflagella, Leptospiral Immunoglobulin-like (Lig) proteins and fibronectin-binding protein.

Leptospira require flagella for efficient colonization and maintenance of infection [11]. These flagella proteins have been found in both pathogenic and nonpathogenic strains of leptospires. Unique morphology *Leptospira* endoflagella are composed of two class of proteins, FlaA and FlaB proteins which are outer membrane sheath and core proteins, respectively [73]. The FlaA sheath is conserved among spirochetes and may help stabilizing FlaB protein helical core into normal shape. In particular, *flaB* gene has been shown to be widely conserved, especially at the N-terminal and C-terminal regions, among the different genera of the family *Leptospiraceae*, spirochetes as well as both Gram-positive and Gram-negative bacteria [73-76].

Leptospiral Immunoglobulin-like protein (Lig) lipoproteins are the major antigen recognized during the acute phase of infection and are virulence factors that play role in host cell attachment, invasion and dissemination from the circulation to different organs and even initiate into deeper tissues during leptospiral invasion [77]. Pathogenic *Leptospira* species but not nonpathogenic species contain the bacterial immunoglobulin superfamily 2 *lig* genes, *lig A* and *lig B* and one pseudogene *lig C* [78, 79]. LigA protein is expressed *in vivo* but not *in vitro* and present only in pathogenic strains. Pathogenic leptospires could established an infection in part by Lig-mediated binding to fibronectin in extracellular matrix or on cell surfaces.

The fibronectin-binding protein may be significant in initial adhesion and invasion at cutaneous or mucosal sites of entry [80]. In addition, *L. interrogans* possesses leptospiral surface adhesin 24 (Lsa24) protein that binds to several extracellular matrix such as laminin, collagens and both cellular and plasma fibronectins [81].

2.2 Host Factors

Host genetics may contribute to the pathogenesis. Lingappa *et al.* [82] found an association between the human leukocyte antigens HLA-DQ6 and the risk of acquiring leptospirosis. Immune-mediated disease has been proposed as one factor influencing the severity of the disease. Activated Kupffer cells release products that are involved in liver damage. These products are superoxide anion, H_2O_2, hydrolytic enzymes, nitrite and nitrate. The overproduction of NO plays a major role in hepatic injury and necrosis [40].

2.3 Chronic or Latent Infection

The possibility of chronic human leptospiral infection is suggested. These patients have no evidence of infection but they are serological test positive. The causative bacteria could be isolated from their specimens both cerebrospinal fluid (CSF) and urine. Uveitis is a chronic condition and it is recognized as a chronic squeal of leptospirosis in humans and horses. Immune involvement in retinal pathology has been demonstrated in horses with spontaneous uveitis [83]. Leptospires have been

isolated from the human eye, and more recently, leptospiral DNA has been amplified from aqueous humor of patients with uveitis [28, 84]. In these cases, uveitis has occurred relatively soon after the acute illness.

3. Protective Immunity

The knowledge of mechanisms of host immunity to *Leptospira* infection or the role of host immunity in leptospirosis is limited. In general, leptospirosis largely depends on humoral immune response.

3.1 Innate immune response

Leptospires are rapidly recognized, engulfed and killed by phagocytes via the complement receptor CR3, or by the complement itself [85-87]. Virulent leptospires require the addition of specific-immunoglobulin of any classes to react with surface LPS determinants before they can be internalized by phagocytic cells either monocytes, monocyte-derived macrophages or neutrophils [11]. The leptopsires are killed by H_2O_2 and defensin [86]. There is no catalase activity found in leptospires. Complement receptor 3 on neutrophils has been reported to serve as the receptor for both pathogenic and nonpathogenic leptospires and acts as adhesion molecule rather than facilitating uptake of the bacteria [85].

The innate immunity recognition relies on interaction of cell surface receptors namely pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) which are present on bacterial cell. These PAMPs consist of molecules such as LPS and peptidoglycans that are not found in the mammalian host cell. The examples of these innate receptors are complement receptors, Toll-like receptor (TLR) family and C-type lectin receptors (CLRs). Werts *et al.* [88] demonstrated that leptospiral LPS activates macrophages via Toll-like receptor 2 (TLR2) and CD14 instead of the more conventional TLR4 like a typical Gram-negative lipopolysaccharide. This may be related to the unique structure of *Leptospira* lipid A, which has a 1-methylphosphate group that differs from lipid A of other bacteria [89, 90]. Recently, Viriyakosol *et al.* [91] examined TLR-4 and demonstrated that leptospiral components other than LPS can induce TLR-4 activation and play a crucial role in protecting mouse from acute lethal infection and control leptospiral burden during sublethal chronic infection. The cytokines from macrophages activated

with *L. interrogans* serovars Icterohaemorrhagie, MIP-2 and IL-6, correlate with leptospiral clearance [91]. However, this report is in contrast to Werts's report. The latter one reported that heat-killed leptospires activate TLR-4 in a CD14-independent manner rather than TLR-2 and CD-14-dependent manner. Recently, Yang *et al.* [92] reported that *L. interrogans* serovars Shermani and Bratislava OMPs, particular LipL32, activated TLR-2 but not TLR-4, resulting in early inflammatory response of renal tubule cells.

3.2 Adaptive immune response

Protective adaptive immunity engenders by antibodies direct against serovar-specific oligosaccharides of LPS. Non-pathogenic and avirulence leptospires are opsonised by natural antibody and killed by phagocytic cells [11]. Broadly reactive and genus-specific antibodies have been reported to associate with OMPs [93]. Lipoproteins and peptidoglycans are also able to trigger the host inflammatory response and can detect antibody against these antigens in infected persons and animals [94, 95].

The role of cell-mediated immunity in leptospirosis is being explored. Several reports showed that Th1 production was the major protective component in leptospiral infection. The γδ TCR+ T cells may play an important role in host defense against *Leptospira in vitro* [96-98]. Pathogenic *Leptospira* can activate monocytes resulting in TNF-α and IL-10 productions [99]. The TNF-α level but not IL-10 correlated with severity of the disease. Peripheral blood mononuclear cell (PBMC) activated by heat-killed whole cell *L. interrogans* induced Th1 response by secreting TNF-α, IFN-γ and IL-12. These cytokines may play an important role in the leptospiral protection [100]. The release of TNF-α by macrophages during leptospirosis plays a dual role in the host response to infections since at lower levels this cytokine is a key element in host defense, but at higher levels it is deleterious in patients with sepsis [41].

4. Immune escape

Leptospirosis is usually an acute disease. However, the unique structural architecture of *L. interrogans* which interact with host immunological processes has the ability to evade the immune system as well, resulting in chronic disease in humans and horse [11]. It is clear that pathogenic leptospires are invasive. They can survive and grow in host tissues by escaping from host defense mechanisms such as complement–mediated killing and phagocytosis [11]**.** However, the mechanism to explain the escape remain elusive. In the absence of specific antibodies, virulent leptospires could be internalized by phagocytic cells, but it is able to survive within neutrophils and macrophages (but not the Kupffer cells) [11]. Monocyte/macrophage-like, Vero cell and Madin-Darby canine kidney cell (MDCK) are permissive host cells for virulent leptospira invasion [2, 101]. Virulent strain leptospires also induce cells apoptosis in macrophages but not on endothelial cells [3].

During the leptospiremic phase, the bacteria are exposed to complement of the alternative pathway of complement but they could withstand complement-mediated destruction and survive in nonimmune cells. Virulent leptospires can protect themselves from host's innate immune response while nonpathogenic strains are engulf and killed by phagocytes. One factor might be due to LfhA which is present only in pathogenic leptospires. This protein can bind to factor H of host, resulting in protecting themselves from the destructive effect of complement activation, especially the alternative pathway of complement activation by inhibiting C3 convertases [61, 102]. After binding to Factor H, C5, C6, C8 and C9 are much less deposited on their surface than nonpathogenic strain. In contrast, nonpathogenic leptospires does not express this protein. They are thus captured and killed by phagocytic cells and complement system.

5. Dendritic Cells

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs). Since they are capable of presenting antigen to naïve T cells and trigger their responses while other APCs can only stimulate memory T cells [103]. Recognition of pathogens by DCs depend on several receptors and one of an important receptor is C-type lectin receptors (CLRs) which recognize the carbohydrate structures on the pathogens.

5.1 Dendritic cells origin

DCs lack of lineage-specific markers such as CD3, CD14, CD16, CD19, which are specific marker for T cells, monocytes, B cells and NK cells, respectively. DCs are heterogeneous family of cells that display differences in localization, cell surface molecules and cellular function [103]. DCs can be generated from both myeloid and lymphoid precursors. DCs generated from $CD34^+$ bone marrow stem cells are common to granulocytes and macrophages and human peripheral blood monocytes, CD14+ cells, in the presence of GM-CSF and IL-4 [104].

Human DCs are characterized by morphology that extend long dendrite, high levels of MHC class II, HLA-DR^{hi} and accessory molecules such as CD40, CD80, CD83, CD86. These molecules support DCs as efficient antigen presentation and costimulation of T cells [103]. DCs are heterogeneous in their expression and level of various markers, and many of these reflect differences in the maturation or activation states. DCs constantly stand as immature form which characterized by high endocytic activity. They sampling pathogens via recognition of PAMPs through PRRs such as CLRs and TLRs and initiation of adaptive immune responses [105-107]. Indeed, DCs are able to distinguish different pathogens through these PRRs. For example, PAMP of bacteria, viruses and parasites, such as LPS, peptidoglycans, CpG motifs, flagella and viral nucleic acids, induce different TLRs signaling which results in dendritic cell maturation. DCs also express wide variety of phagocytic receptor such as Fc receptors, complement receptors, mannose receptors and scavenger receptors [108, 109].

The surface phenotype as well as the functional attributes of DCs change according to their stage of activation [103]. An immature stage express high level of surface receptors potentially involved in antigen capture, including the

mannose receptor, DC-SIGN and the immunoglobulin Fc receptors and has low T-cell stimulating activity. In contrast, mature DCs reduce antigen uptake functions as decrease antigen capture receptors and dramatically increase their antigen-presenting capacity by increase expression of MHC-peptide complexes.

Immature DCs capture pathogens in the peripheral tissues, degrade them to small peptides and migrate to the draining lymph nodes. Subsequently, DCs become mature and present processed fragments onto their surface using MHC molecules. Mature DCs undergo cytoskeleton rearrangement that lead to the inhibition of the phagocytic activity and are programmed to apoptotic death. At the same time, they also stimulate co-stimulatory molecule expression such as CD80, CD83 and CD86 and these act as co-receptors for T cell activation. They also upregulate CCR7 chemokine receptor which induce DCs migrate from blood stream to lymph nodes to initiate T and B cells responses as shown in Figure 4 [110]. Several substances such as LPS, bacterial DNA or double-strand RNA, pro and anti-inflammatory cytokines including TNF-α, IL-1, IL-6, IFNs and IL-10 are also known to induce maturation of DCs [111]. In addition, binding of Fc- and complement-receptors have some role in DCs maturation by specific uptake, processing and presentation of opsonised antigen [108].

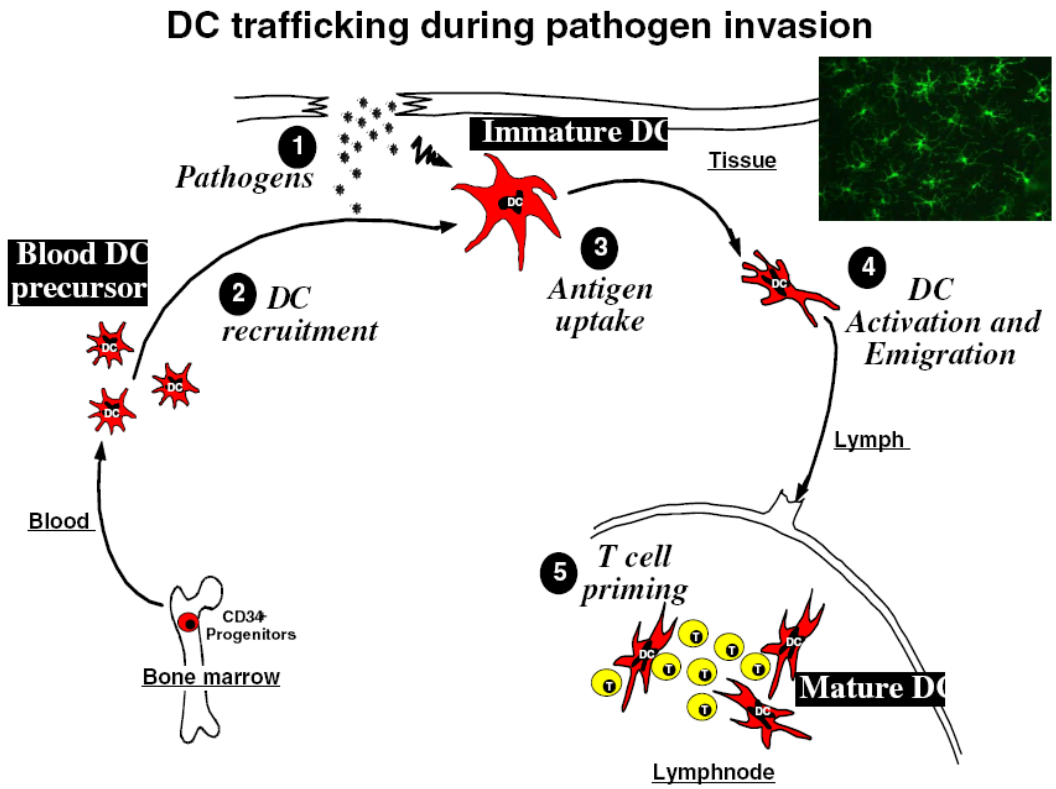

Figure 4 DCs Life Cycle [112]. Circulating DCs enter tissues as immature DCs, directly encounter with pathogens and then migrate to lymphoid organs and become mature. Mature DCs present peptide-MHC complexes and activate T cells.

5.2 Dendritic cell functions

DCs are present throughout the body at sites of antigens exposure such as mucosal tissues, thymus, lung, spleen, tonsil, T cells area of lymph nodes and blood to act as sentinels, processing and presenting antigens to elicit appropriate immune responses [103]. Human DCs also occur in normal skin which is the large part of antigen contact of the body [113].

DCs express several PRRs such as TLRs and CLRs, including macrophage mannose receptor (MMR) and DC-SIGN. CLRs recognition is highly dependent on the density of PAMPs on pathogen surface as well as the degree of multimerization of the lectin receptors. The main function of CLRs is binding and internalizing antigens for degradation, thus enhancing antigens processing and

presentation. At the same time, lysosomal degradation produces antigenic fragments that complex with MHC molecules at the cell surface and then staring to stimulate the adaptive immune response [114]. In contrast, TLRs recognize several type of foreign antigens such as proteins and carbohydrate structures then trigger intracellular signaling cascades that lead to production of proinflammatory cytokines and to activation of T cells. CLRs have been reported of cross talk with TLRs. This cross-talk fine tunes the balance immune activation. For example, binding of mycobacterium components to DC-SIGN receptor and TLRs might skew the immune response from Th1 toward Th2 response thus facilitate immune escape of mycobacterium [115].

DCs regulate immune response by directing antigen-specific T cells to die, to become anergic, effector or memory T cells [116-118]. The function of DCs at immune privileged sites such as eye is tolerogenic rather than immunogenic [117]. DCs can induce apoptosis of T cells to maintain homeostasis by Fas and FasL [116]. The induction of cell anergy by DCs might be due to incomplete maturation, low costimulatory molecules expression or influence of anti-inflammatory cytokines such as IL-10 and TGF-β [118]. DCs also play an important role in selection the type of immune response by polarize naïve T cells toward either Th1 or Th2 or regulatory T cells (Treg) [119]. This response depends on the binding of pathogen PAMPs and PRRs on DCs in the immature stage and resulting in selective programming DC during their maturation.

However, pathogens have established numerous strategies to evade DCs initial immune response by interfere at several steps such as interfere with generation and survival of DCs, interfere antigen-presenting mechanisms, inhibit T cell proliferation and differentiation [120].

5.3 Dendritic cell-T cell interactions

Upon DCs activation, mature DCs express costimulatory molecules and produce various cytokines, activate naïve CD4+ T cells that undergo a complex differentiation program and generate an antigen-specific primary T cells response[111]. The differentiation of naïve T cells into Th1 or Th2 effector is influenced by the type of microorganism recognized, the cytokines present at the site of the activation, the nature and amount of signals as TCR-MHC complexes,

costimulatory molecules expression and cytokines produce by DCs, the genetic background and the type and the activation state of the DCs [111, 121].

T cell priming is then followed by the activation of primary B cell response resulting in both cellular and humoral immune response. Th1 produces high levels of IFN-γ whereas Th2 produces high levels of IL-4 that crucial for induction of cell mediated and humoral immune responses, respectively. On the same time, DCs also control the function and expansion of regulatory T cells (Treg) which has potential to suppress the proliferation and production of IFN-γ producing cells [122].

6. Dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN) or CD209

Classical CLRs contain carbohydrate recognition domain (CRD) that interacts with specific carbohydrate structures in a calcium (Ca^{2+})–dependent manner. Ca^{2+} ions are directly involved in both ligand binding as well as maintaining the CRD structure [123]. An important CLR that functions as prototypic receptor for CLR family is DC-SIGN (DC-Specific ICAM-3-Grabbing Non-integrin) which is also known as CD209. DC-SIGN is abundantly expressed on the surface of DCs and IL-4 treated monocytes [4, 124]. It is also expressed on macrophages of the lung alveolae, placenta and inflammatory lesion [125, 126]. This receptor was identified in 2000 by Yvette van Kooyk *et al.* [4]. DC-SIGN expresses in different levels of clustering on DCs surface depending on their differentiating state when developed from monocytes precursor [127]. During development of human monocytes-derived DCs, DC-SIGN molecules distribution alters from random-distribution into well-defined microdomains.

6.1 DC-SIGN structure

DC-SIGN is a 44 kDa type II transmembrane protein which consists of extracellular domain, transmembrane and cytoplasmic region Figure 5. The extracellular domain contains one CRD that interacts with specific carbohydrate structures. The CRD of DC-SIGN is a globular structure and forms two loop for Ca^{2+} binding sites. One site is essential for the conformation of the CRD, and the other is essential for direct coordination of the carbohydrate structures. Four amino acids (Glu347, Asn349, Glu354 and Asn365) interact with Ca^{2+} at this site and dictate the

recognition of specific carbohydrate structure. The neck domain made up from seven complete and one incomplete, 23-residues tandem repeats that involve in tetamerization of the receptor which regulates carbohydrate specificity. Transmembrane region is essential in localization of DCSIGN on cell surface. The cytoplasmic tail containing a tri-acidic cluster of amino acids as well as internalization motifs such as di-leucine (Leu-Leu) and tri-acidic which mediating endocytosis [128, 129].

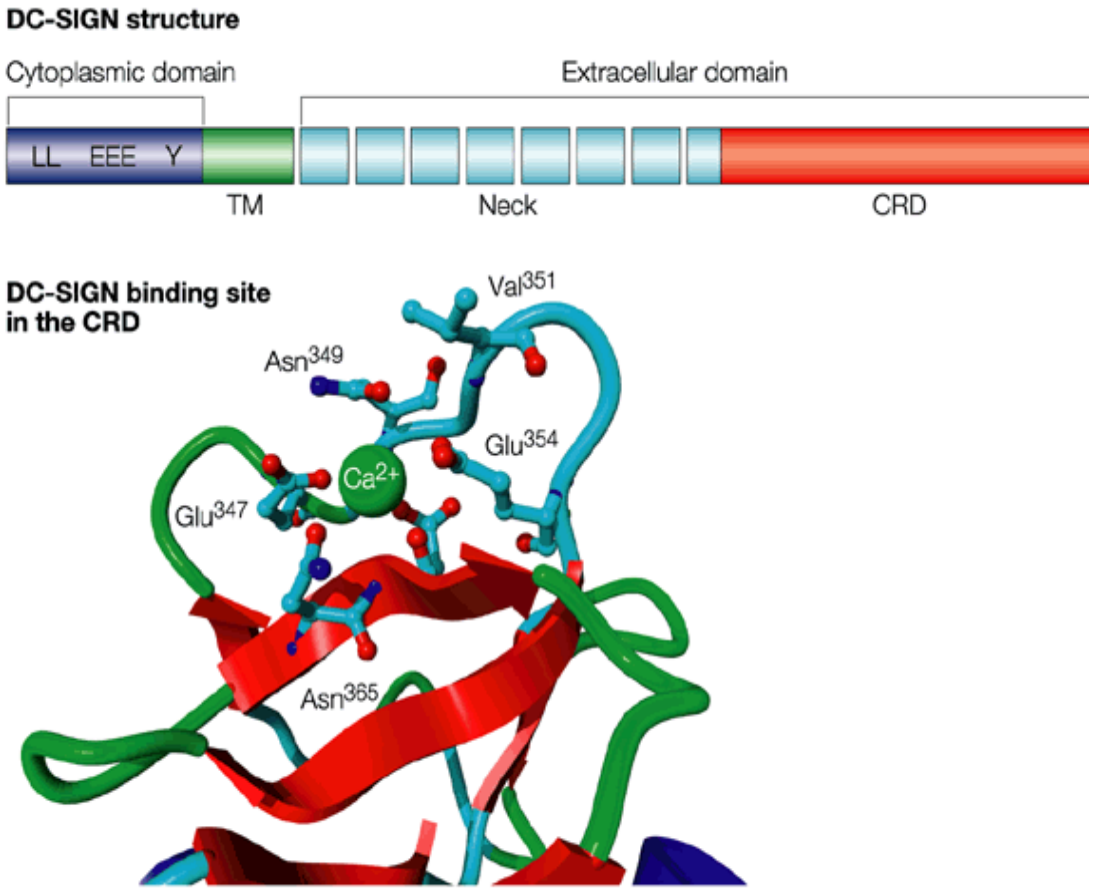

Figure 5 DC-SIGN Structure [130].

6.2 DC-SIGN functions

DC-SIGN has high affinity for high-mannose glycans as well as fucose-containing Lewis antigens such as Le^x, Le^y, Le^a and Le^b , indicates that the carbohydrate specificity of DC-SIGN are broad [6-10, 131]. DC-SIGN plays many functions on DC activation immune responses. First, DC-SIGN capture and internalize antigens into DCs leading to the increasing expression of the co-stimulatory molecules on the surface of DC cell membrane [114]. Second, DC-SIGN is able to bind high mannose structure in ICAM-3 and establishes the initial contact between DCs and T cells through ICAM-3 and exerted immunosynapse formation that allows T cell receptor to scan the surface of DCs and T cells and then differentiation into either Th1 or Th2 cells [4, 5]. This interaction prolongs cell-cell contact and thereby prolonging TCR signaling. Third, through ICAM-2 the DC-SIGN could mediate DC migration to both peripheral tissues and secondary lymphoid tissues [132].

DC-SIGN has been reported to cross talk with TLR2, leading to better activation of NF-κB [133]. These enhancement occurs by increasing viral concentrate on the cell surface which facilitates TLR2 signaling.

6.3 DC-SIGN and immune escape

Several viruses bind DC-SIGN and internalizes into target cells for infection such as Dengue virus, HCMV, HCV, Ebola and SARS [7, 115, 130, 134]. DC-SIGN receptor could be the target of certain viruses which may lead to escape of immune surveillance or immune suppression. For example, HIV-1 and HCV bind DC-SIGN to escape lysosomal degradation and to gain access to CD4+ T cells that are primary target of HIV-1 by increase DC-SIGN expression for immunological synapse. formation that enhance viral transmission. In addition, DC-SIGN also modulates immune responses to several other pathogens. Mycobacterium component, ManLAM, which bind DC-SIGN was shown to modulate DC function by inhibiting DC maturation, preventing IL-12 secretion and inducing IL-10 production [115]. These processes may shift the immune balance to benefit pathogen survival. *Helicobacter pylori* also use DC-SIGN to escape immune response by blocking Th1 induction and thus allowing induce immune response toward Th2 [135]. Probiotic bacteria as *Lactobacillus* strains also cause immune suppression by activate Treg and IL-10

production through engagement of DC-SIGN on DCs but did not inhibit DC maturation [136].

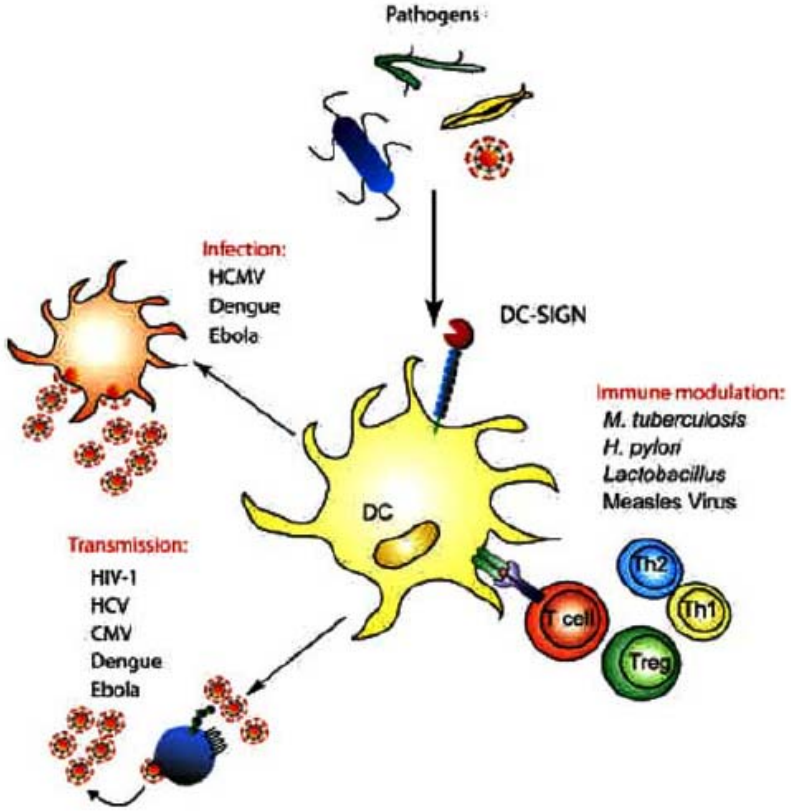

Figure 6 The initial interaction of pathogens with DCs determines the immunological outcome [137].

CHAPTER IV
MATERIALS AND METHODS

1. Bacteria

L. interrogans serovar Autumnalis L-643 and BL-6 strains were isolated from patient who died from leptospirosis and from recoverd leptospirosis patient, respectively. These bacteria were kept in liquid nitrogen tank and avoid to passage more than 10 times before performing the experiment. Both isolates were collected from Regional Hospital, Loi province, Thailand. *L. interrogans* serovar Pyrogenes 2317 was collected from patient and attenuated by subculturing. The virulence of *L. interrogans* serovar Pyrogene 2317 was kindly confirmed in a hamster model by Department of Veterinary Medicine, at Royal Thai Army-Armed Forces Research Institute of Medical Sciences, Thailand. The virulence was tested by inoculating $0.5x10^8$ cells/ml of leptospires to a group 4-weeks old specific pathogen free (SPF) female hamsters (4 SPF/group) and observing clinical symptoms until the time of death (usually within 5 days). The virulent leptospira caused severe disease and death while avirulent leptospira caused mild symptoms of illness. Reference leptospires kindly provided by the National Institute of Animal Health (NIAH), Department of Livestock Development, Ministry of Agricultural and Cooperatives, Thailand and the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand are shown in Table 1. Leptospires were grown in liquid Ellinghausen McCullough-Johnson-Harris (EMJH) medium supplemented with 3% rabbit serum and incubated at 30^{o}C. *Salmonella typhimurium* was cultured in LB broth and used as positive control for leptospiral binding assay. The reference leptospires used for preparation of secreted and whole cell antigens were grown for 7 to 10 days. The organisms were pelleted by centrifugation at 10,000 rpm (Superspeed centrifuge, Sorval, Model RC28S) for 15 min at room temperature and washed 3 times with 0.01 M phosphate buffer saline (PBS), pH 7.2. The antigens were kept in small aliquots at -20 ^{o}C. The whole cell antigen was killed by treating leptospires with 0.02% formalin

in PBS for 18 h at 4 ^{o}C. These cells were pelleted and washed 3 times with 0.15 M PBS pH 7.2 and kept at -20 ^{o}C.

For the secreted antigen preparation, the pellet cells were resuspended in PBS incubated overnight at 30 ^{o}C with gentle agitation and then centrifuged at 10,000 rpm for 15 min at room temperature to sediment the organism.

1.1 Biochemical component of secreted antigens assay

Sodium dodecyl sulfate-polyacrelamide gel electrophoresis (SDS-PAGE) was performed with 12% (wt/vol) resolveing gel and 3.5% (wt/vol) stacking gel. Secreted antigens were identified components by treated with either proteinase K or sodium periodate. For protein treatment, antigens were treated with 10 μg/ml proteinase K enzyme (Promega, Madison, USA) at 60 ^{o}C for 1 h and then inactivated by heated in a boiling water bath for 5 minutes. For carbohydrate treatment, antigen was treated with 0.01 M sodium metaperiodate (Sigma, Steinheim, Germany) for 18 to 24 h at 4 ^{o}C. Then, secreted antigens were solubilized in sample loading buffered and loaded onto gel and run at 80 vol and stained with either Coomassie brillian blue or silver nitrate. The low molecular weight protein was used as standard marker.

1.2 Protein concentration of antigens

Leptospiral whole cells and secreted antigens were measured by Bradford protein assay (Bio-Rad, CA, USA). Bovine serum albumin (BSA)(Sigma, St. Louise, MO, USA) was used as the standard proteins. Two serial dilutions of standard proteins ranging from 1.25 to 10 μg/ml were prepared according to microassay procedure. Then, 800 μl of each standard dilution and sample solution were added with 200 μl of dye reagent, mixed and incubate at room temperature for 30 minutes, and measured OD at 595 nm. Both whole cells and secreted antigens preparations were used for biotynilated lectin ELISA.

Table 1 Reference leptospira serogroups used for preparation of secreted and whole cell antigens.

No.	Serogroup	No.	Serogroup	No.	Serogroup
1	Australis	9	Djasiman	17	Panama
2	Autumnalis	10	Grippotyphosa	18	Pomona
3	Ballum	11	Hardjo	20	Sarmin
4	Bataviae	12	Hebdomadis	21	Sejroe
5	Bratislava	13	Icterohaemorrhagiae	22	Tarassovi
6	Canicola	14	Javanica	23	Wolffi
7	Celledoni	15	Louisiana	24	Samatanga
8	Cynopteri	16	Mini		(Biflexa Patoc I)

2. Cell lines

K-562 cells were cultured in RPMI 1640 complete medium supplemented with 10% FBS (Sigma, St. Louise, MO, USA) as described previously [131]. K-562 DC-SIGN transfected cells (K-SIGN) were cultured in 25% RPMI 1640 (Gibco, NY, USA), 75% ISCOV's DMEM medium (Gibco, NY, USA) supplemented with 10% FBS (Sigma, St. Louise, MO, USA) and 400 μg/ml G418 (Gibco, NY, USA). These cells were kindly provided by Dr. Yvette van Kooyk, Vrije University Medical Center Amsterdam, The Netherlands. The level of DC-SIGN expression was performed by staining with 20 μl anti-DC-SIGN, AZN-D1, hybridoma supernatant (8 μg/ml) and goat-anti mouse-FITC (BD BioScience, San Jose, CA, USA) and analyzed by FACS Calibor (Becton Dickinson).

3. Monocytes derived dendritic cells (MoDCs)

Peripheral blood was obtained from buffy coats of healthy members of the Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University and USMAC-Armed Forces Research Institute for Medical Science, Thailand. Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation using Histopaque (Sigma, St. Louise, MO, USA) at blood:RPMI:Histopague ratio of 1:1:1. $CD14^+$ monocytes were isolated using CD14 MACS MicroBeads (Miltenyi Biotec, Auburn, CA, USA). MoDCs were generated by culturing monocytes in RPMI 1640 complete medium containing 10% FCS (BioWithaker, Cambrix, UK) in presence of 100 ng/ml rhIL-4 and 100 ng/ml rhGM-CSF (both cytokines from R&D Systems Inc., Mineapolis, MN, USA) for 5–7 days [5].

4. Lectin binding assay

Lectins are complex multidomain proteins that specifically interact with carbohydrate with different specificity depending on glycans structure (Table 2). Natural ligands are presumably high N-linked glycans. This property provides suitable tool used in screening carbohydrate components on many organisms. Then, biotinylated lectin binding assay was performed by ELISA. Formaldehyde killed leptospires at concentration 10^7 cells in 0.2 M carbonate buffer, pH 9.6 were coated in Maxisorp plate (50 µl/well) at 37 ^oC for 2 h and blocked with 5% skim milk in Tris-saline magnesium buffer (TSM) pH 8.0 for 1 h at 37 ^oC. Then, 50 µl of biotinylated lectins which are Concanavalin A (ConA), *Dolichos biflorus* (DBA), Peanut agglutinin (PNA), *Ricinus communis* agglutinin (RCA_{120}), Soybean agglutinin (SBA), Wheat germ agglutinin (WGA) and *Ulex europaeus* agglutinin (UEA1) (Vector Laboratories Inc., CA, USA) were added and incubated for 1 h at 37 ^oC. Unbound biotinylated lectins were washed away with TSM. Then, 50 µl streptavidin-alkaline phosphatase (ST-AP, Southern Biotectnology Assocociated Inc., AL, USA) at dilution 1:1000 were added and incubated for 1 h at 37 ^oC and washed 3 times with TSM containing 0.05% Tween 20 (TSM/T). The binding was determined by adding diethanolamine/*p*-nitrophenyl phosphate (DNPP, Pierce, IL, USA) and measured OD at 405 nm. The positive control well was coated with 100 µg/ml mannan (Sigma, St. Louise, MO, USA) and the reaction was detected with biotinylated ConA conjugate.

Table 2 Carbohydrate – Binding specificity of the lectins use in this study [138].

Lectins	Glycan ligands
ConA	branch αmonosidic high mannose
	N-linked glycans, Glu
DBA	GalNAcα1-3GalNAc
	GalNAcα1-3
PNA	Gal, Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)
RCA_{120}	Galβ1-4GlcNAcβ1-R
SBA	terminal α,βGalNAc, α,βGal
UEA1	Fucα1-2Gal-R
WGA	Sialic acid / N-acetylglucosamine
	GlcNAcβ1-4GlcNAcβ1-4GlcNAc

5. Soluble DC-SIGN-Fc adhesion assay

This ELISA assay utilized the DC-SIGN-Fc chimeric protein composing of the extracellular portion of DC-SIGN (amino acids 64–404) fused at the C terminus to a human IgG1 Fc fragment as previously described [139]. Killed leptospires at concentration of 10^7 cells or 50 μl whole cell antigens (10 μg/ml) or secreted antigens (5 μg/ml) were coated onto either polyvinyl or Maxisorp plate (50 μl/well) and incubated overnight at 4 °C or 2 h at 37 °C and washed 3 times with TSM. The coated plate was blocked with 100 μl of 5% skim milk for 1 h at 37 °C and washed 3 time with TSM. Then, 50 μl of soluble DC-SIGN-Fc (1:1 in TSM) were added and incubated for 1 h at 37°C. This soluble DC-SIGN-Fc was kindly provided by Dr. Yvette van Kooyk (Vrije University Medical Center Amsterdam, The Netherlands). Then, 50 μl of rabbit anti-human IgG-conjugate (Southern Technology Associated Inc., AL, USA) at dilution 1:1,000 were added and incubated for 1 h at 37°C, washed 3 times with TSM/T. ST-AP (Southern Biotectnology Assocociated. Inc., AL, USA) at dilution 1:1000 were added 50 μl and incubated for 1 h at 37°C and washed 3 times with TSM/T. The binding were detected by DNPP substrate (Pierce, IL, USA) and measured OD at 405 nm. Binding specificity was determined by preincubated soluble DC-SIGN-Fc with mannan (50 μg/ml) for 15 min at room temperature. The positive control well was coated with mannan 100 μg/ml.

6. Bacteria binding

Bacteria at concentration of 10^9 cells/ml were labeled with 50 μl FITC (1 mg/ml, Sigma, St. Louise, MO, USA) for 1 h at room temperature and followed by extensive washing. Some of them were collected and killed with 2% paraformaldehyde for 30 min at room temperature before use as whole cell antigens. These labeled leptospires were aliquoted into small tubes, kept at 4 °C and used within 3 months. The labeling was confirmed under fluorescent microscope.

Labeled leptospires were added to cell lines or MoDCs at various ratios (1:10, 1:100 and 1:200 cell/bacteria) and incubated for 40 min at 37 °C. Samples were

analyzed using flow cytometry and determined by measuring mean fluorescent intensity (MFI). Specificity was determined in the presence of anti-DC-SIGN antibodies (AZN-D1) 20 μg/ml [131], mannan (50 μg/ml) or EDTA (10 mM) for 20 min at room temperature before adding bacteria.

7. DCs maturation

Immature DCs $5x10^4$ cells were incubated with paraformaldehyde killed leptospiral $5x10^6$ (at ratio MoDCs:leptospira 1:100) or 100 μl *E.coli* LPS (5 μg/ml, Sigma, St. Louise, MO, USA) in RPMI 1640 complete medium plus 10% FCS (BioWithaker, Cambrix, UK). After 2 days of incubation, DCs were harvested and stained cell-surface DC maturation markers CD83-FITC and CD86-PE (BD Bioscience, San Jose, CA, USA). The cells were analyzed by flow cytometry. The data were presented as mean fluorescent intensity (MFI).

8. Cytokines assay

The presence of IL-10, IL-12p70 and TNF-α in supernatants of the two days cultured leptospiral-MoDCs were determined by ELISA according to the manufacturer's instructions (R&D Systems, Mineapolis, MN, USA). Briefly, 50 μl/ml assay diluent and 200 μl/ml culture supernatant or serial dilution of standard controls were added to coated wells and incubated at room temperature for 2 h. Wells were washed 3 to 4 times with washing buffer, added 200 μl/ml conjugate, incubated at room temperature for 1 h for TNF-α and IL-10 or 2 h for IL-12p70. After washing, 200 μl/ml substrate solution was added, incubated for 20 min. Then, 50 μl/ml stop solution was added and measured OD at 450 nm within 30 min. The values were obtained by extrapolation using the values obtained with standard amounts of recombinant cytokines.

9. T cells activation

Allogeneic naïve T cells were obtained from PBMC of healthy donor through negative selection which removed B, NK and memory T lymphocytes by E-Rosetting with neuraminidase treated sheep red blood cells (SRBC). Briefly, 12 ml SRBC were

centrifuged at 1000xg for 10 min. One ml neuraminidase (1 U/ml, Sigma, St. Louise, MO, USA) was added to the cell pellet, mixed and incubated for 1 h at 37 ^{o}C. Cells were washed one time with RPMI and resuspended in 49 ml RPMI 1640 supplemented with 10% FBS. Then, 2 ml of treated SRBC together with 2 ml FBS was added to 1 ml suspension of 10^7 PBMC (to make ratio 2:2:1 SRBC/FBS/PBMC) and incubated for 10 min at 37 ^{o}C. Then, the SRBC/FBS/PBMC suspension was placed on ice for 1 h before adding onto Histopaque (Sigma, St. Louise, MO, USA) at ratio 3:10 (Histopaque: SRBC/FBS/PBMC). Cells were collected for E-rosette positive present at the bottom of tube. These cells were lysed with 1 ml of 0.83% NH_4Cl lysing buffer (Otho Diagnostic System, UK) for 5 min at room temperature and washed 2x with RPMI. The cell pellet was further purified for naïve T cells by sorting negative for CD8, CD14, CD20, CD56, CD45RO and gamma-delta by flow cytometry.

For T cell differentiation, MoDCs were cocultured with leptospiral (100 bacteria/cells) for 2 days and incubated with naïve T cells (5,000 DCs /20,000 T cells) for 12 to 14 days at 37 ^{o}C in CO_2 incubator with 5% CO_2 atmosphere. Either 5 μg/ml *E. coli* LPS (Sigma, St. Louise, MO, USA), 100 μg/ml polyI:C (Invitrogen, CA, USA) or 100 μg/ml LPS/prostagrandin E2 (PGE_2, Invitrogen, CA, USA) were used as positive controls. To determine the intracellular cytokine production, T cells were re-stimulated with 10 ng/ml PMA (Sigma, St. Louise, MO, USA) and 1 μg/ml ionomycin (Sigma, St. Louise, MO, USA) for 6 h. The last 5 h after incubation, 25 μl Golgi Plug blocking agent (at dilution 1:50 in RPMI, BD Bioscience, CA, USA) were added. Intracellular IL-4 and IFN-γ cytokines were determined by intracellular staining using anti-IL-4-PE and anti-IFN-γ-FITC (both cytokines from BD Bioscience, San Jose, CA, USA) and analyzed by flow cytometry.

10. Statistical analysis

Data are presented as mean±S.E. of duplicate samples of the independent experiments.

CHAPTER V
RESULTS

1. Expression of carbohydrates on *L. interrogans*

Leptospiral LPS is the major component that presents on the surface of bacteria. It is highly immunogenic and is responsible for serovar specificity. The epitopes for serovar specificity are small oligosaccharides derived from the polysaccharides of LPS. However, the chemical composition of the leptospiral polysaccharide has been analyzed in only few strains such as *L. interrogans* serovars Copenhageni and Hardjo and *L. borpetersenii* serovars Hardjo [11, 39]. These leptospires have rhamnose and mannosamine as predominant sugars similar with other leptospires [39]. Moreover, *L. biflexa* serovar Patoc showed common backbone structure of mannose complex that has similar repeating unit and cross-react with the antisera raised against pathogenic strains [140-142]. In this study, we analyzed the carbohydrate components on leptospires using a panel of biotinylated plant lectin with well-characterized carbohydrate binding specificity.

Whole cells of twenty two reference pathogenic *L. interrogans* serogroups and nonpathogenic *L. biflexa* strain Patoc I were screened for carbohydrate components by ELISA using biotinylated plant lectins shown in Table 1. The results showed that leptospires contained different carbohydrate components since all serogroups showed differences in lectin recognition (Table 3 and Figure 7 (A-D), mean ± S.E., n = 2). All of the tested leptospires were recognized by ConA and RCA1. However, some serogroups were recognized by WGA, SBA, DBA and UEA1. These evidences suggest that the common carbohydrate on *Leptospira* composed of high mannose complex, N-glycan and Galβ1-4GlcNAcβ1-R. The other minor components are GlcNAcβ1-4GlcNAcβ1-4GlcNAc, terminal GalNAc, GalNAcα1-3GalNAc and Fucα1-2Gal-R.

Virulent and avirulent *L. interrogans* serovar Pyrogenes 2317 as well as *L. interrogans* serovar Autumnalis L-643 and BL-6 isolated from patients, were further investigated. As shown in Figure 7 E, all leptospires reacted to a certain extent with the plant lectins, ConA; RCA_{120} and WGA, that reacted strongly with high mannoses, Galβ1-4GlcNAcβ1-R and GlcNAcβ1-4GlcNAcβ1-4GlcNAc, respectively. These results indicated that either reference strains or clinical isolated strains possess the potential molecules that might interact with DC-SIGN.

Table 3. Lectin binding assay for analyzing the sugar component on 22 reference pathogenic leptospira serogroups and a nonpathogenic *L. biflexa* strain Patoc I

The results are showed as mean ± S.E. of duplicate wells of two independent experiments.

	Serogroup	**OD 405 nm**						
		ConA	SBA	DBA	WGA	UEA1	RCA1	PNA
1	Australis	2.459	0.491	0.235	0.231	0.225	2.041	0.266
2	Autumnalis	2.160	0.461	0.197	0.844	0.227	1.358	0.216
3	Ballum	2.089	0.479	0.240	0.952	0.285	1.205	0.221
4	Bataviae	2.821	0.324	0.396	0.207	0.251	1.938	0.168
5	Bratislava	1.748	0.271	0.204	0.655	0.317	1.352	0.188
6	Canicola	1.509	1.005	0.222	0.472	0.292	1.665	0.187
7	Celledoni	2.851	0.713	0.486	0.694	0.293	1.762	0.694
8	Cynopteri	1.757	0.285	0.121	0.431	0.289	0.828	0.244
9	Djasiman	2.382	0.527	0.195	0.651	0.220	1.562	0.151
10	Grippotyphosa	1.943	0.377	0.150	0.300	0.115	1.304	0.157
11	Hardjo	1.611	0.295	0.196	1.202	0.129	1.216	0.225
12	Hebdomadis	1.557	0.278	0.171	1.102	0.157	1.238	0.120
13	Icterohaemorrhagiae	2.119	0.639	0.127	0.738	0.161	1.394	0.291
14	Javanica	1.920	0.439	0.075	0.125	0.096	1.035	0.078
15	Louisiana	2.035	0.429	0.105	0.698	0.149	1.392	0.081
16	Mini	2.588	0.608	0.373	0.167	0.209	1.483	0.148
17	Panama	1.508	0.433	0.150	0.931	0.092	1.428	0.169
18	Pomona	1.926	0.581	0.354	0.209	0.194	1.495	0.169
19	Sarmin	2.373	0.532	0.157	0.761	0.359	1.699	0.211
20	Sejroe	1.932	0.500	0.259	0.985	0.049	1.382	0.225
21	Tarassovi	1.829	0.713	0.631	0.949	0.560	1.730	0.202
22	Wolffi	1.720	0.324	0.210	0.772	0.323	1.168	0.354
23	Samatanga	2.029	0.346	0.184	0.591	0.325	0.775	0.212

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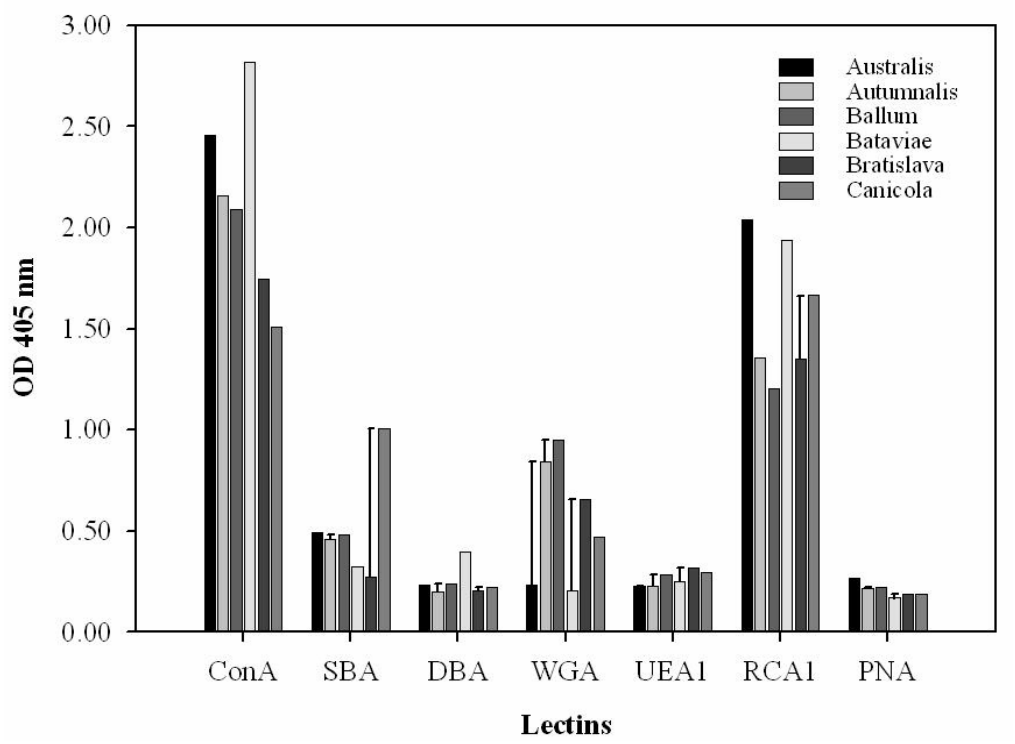
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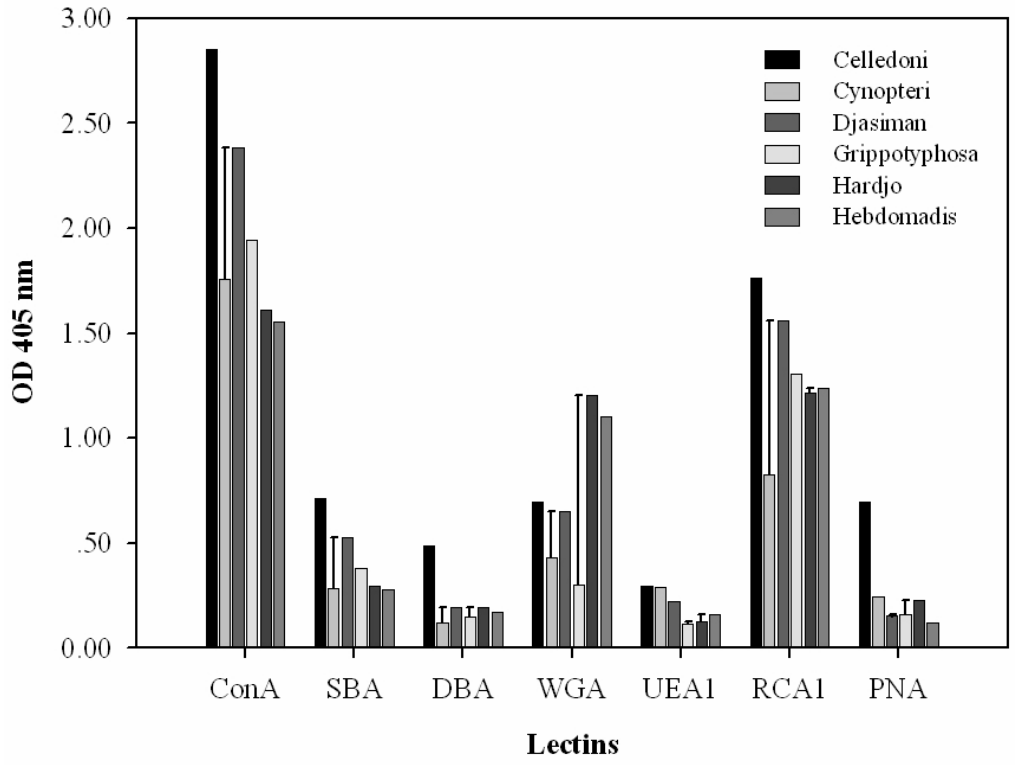
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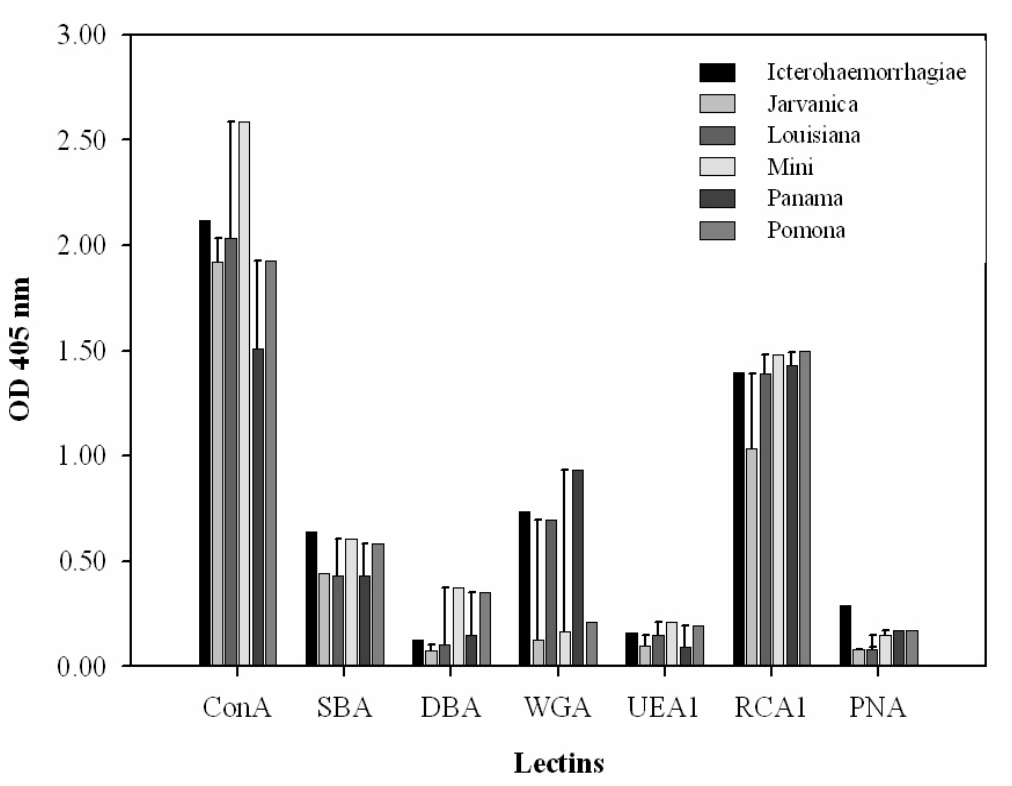
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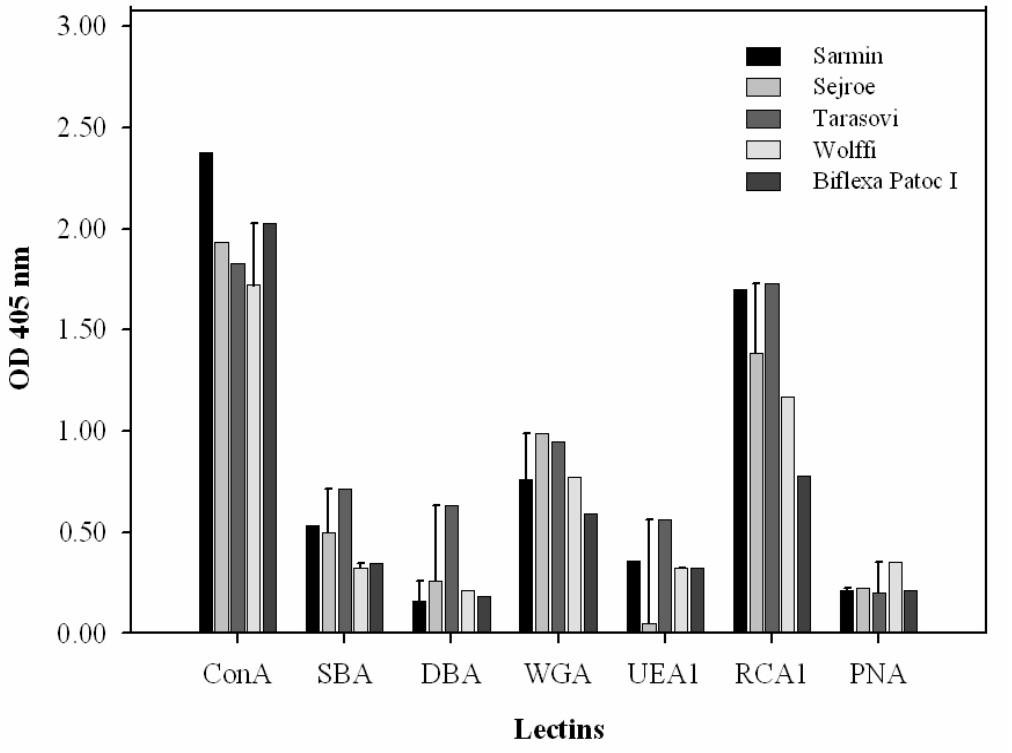
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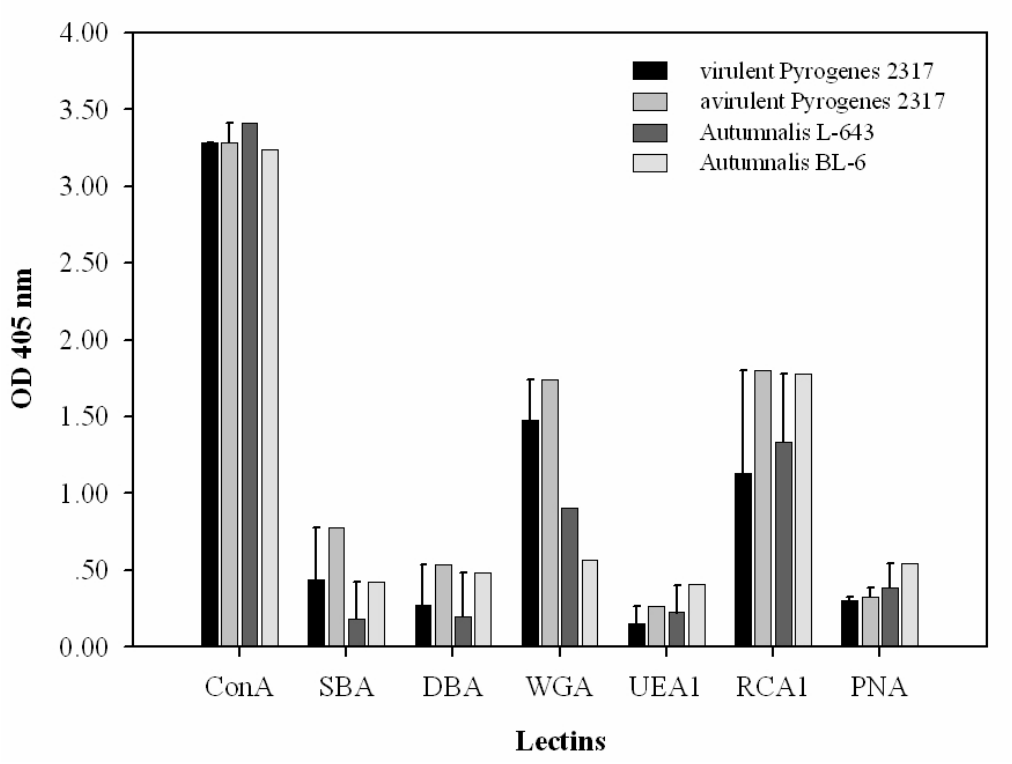


Figure **7** ELISA lectin binding assay for carbohydrate component of reference pathogenic leptospiral serogroups (A-D), a nonpathogenic *L. biflexa* serovars Patoc I (D) and four clinical isolates (E). *L. interrogans* coated microtitter plate were determined for the carbohydrates on their cell surface using biotinylated lectins and measured OD at 405 nm after adding substrate.

Secreted antigens were identified components by SDS-PAGE and stained with Coomassie brillian blue and silver nitrate (Figure 8 (A-E) and 9 (A-E), respectively) as described in Material and Methods (1.1 Biochemical component of secreted antigens assay). The SDS-PAGE profile of control untreated antigens (lane 2: sodium periodate, lane 4: proteinase K) and treated antigens (lane 3: sodium periodate, lane 5: proteinase K). The results showed that leptospiral secreted antigens contains carbohydrate components.

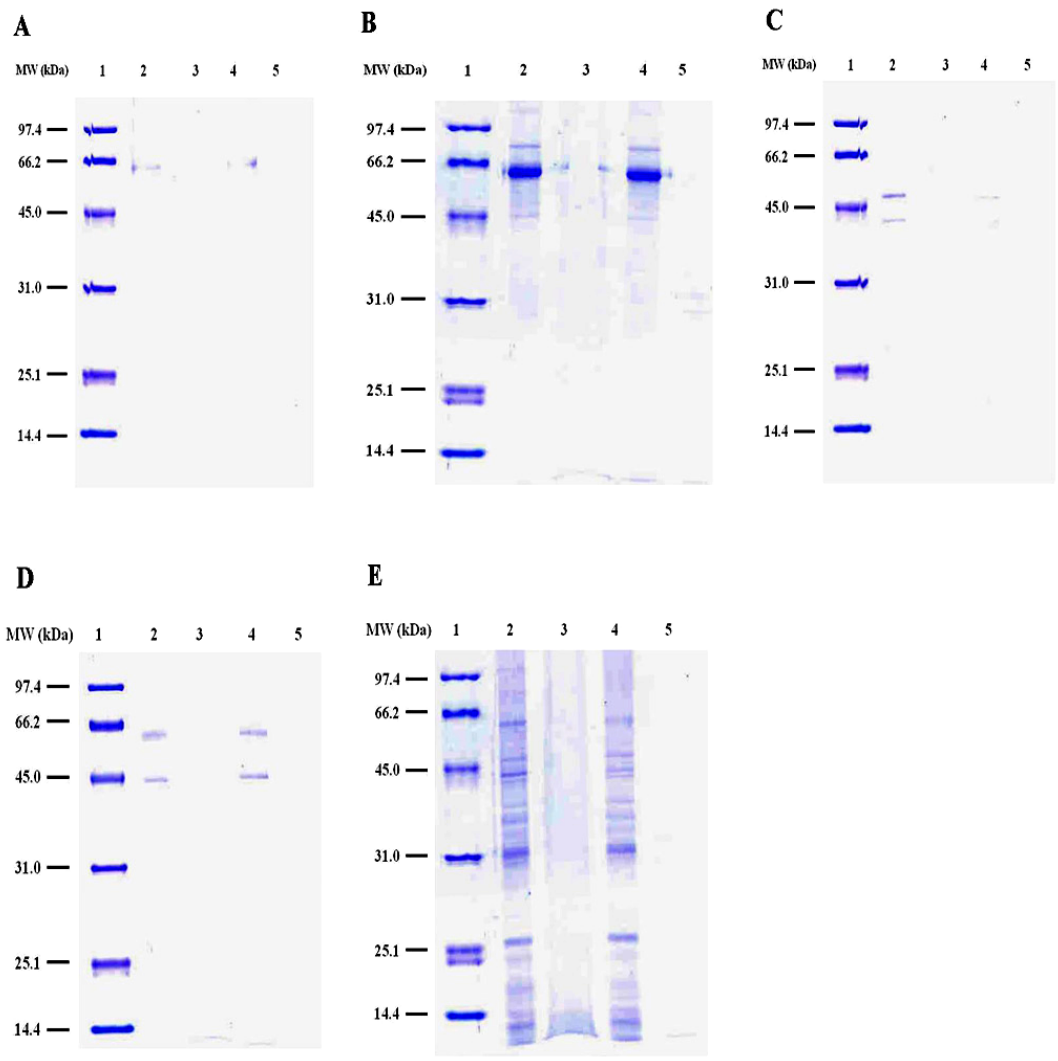


Figure 8 Coomassie brillian blue SDS-PAGE of secreted antigen (SC) prepared from reference *L. interrogans* serovar Autumnalis (A), Bataviae (B), Javanica (C), Pyrogenes (D) and Sejroe (E). Molecular marker (MW) is shown on the left hand.

Lane 1: Molecular marker

Lane 2: Control Antigen for sodium periodate treatment

Lane 3: Antigen treated with sodium periodate

Lane 4: Control Antigen for proteinase K treatment

Lane 5: Antigen treated with proteinase K

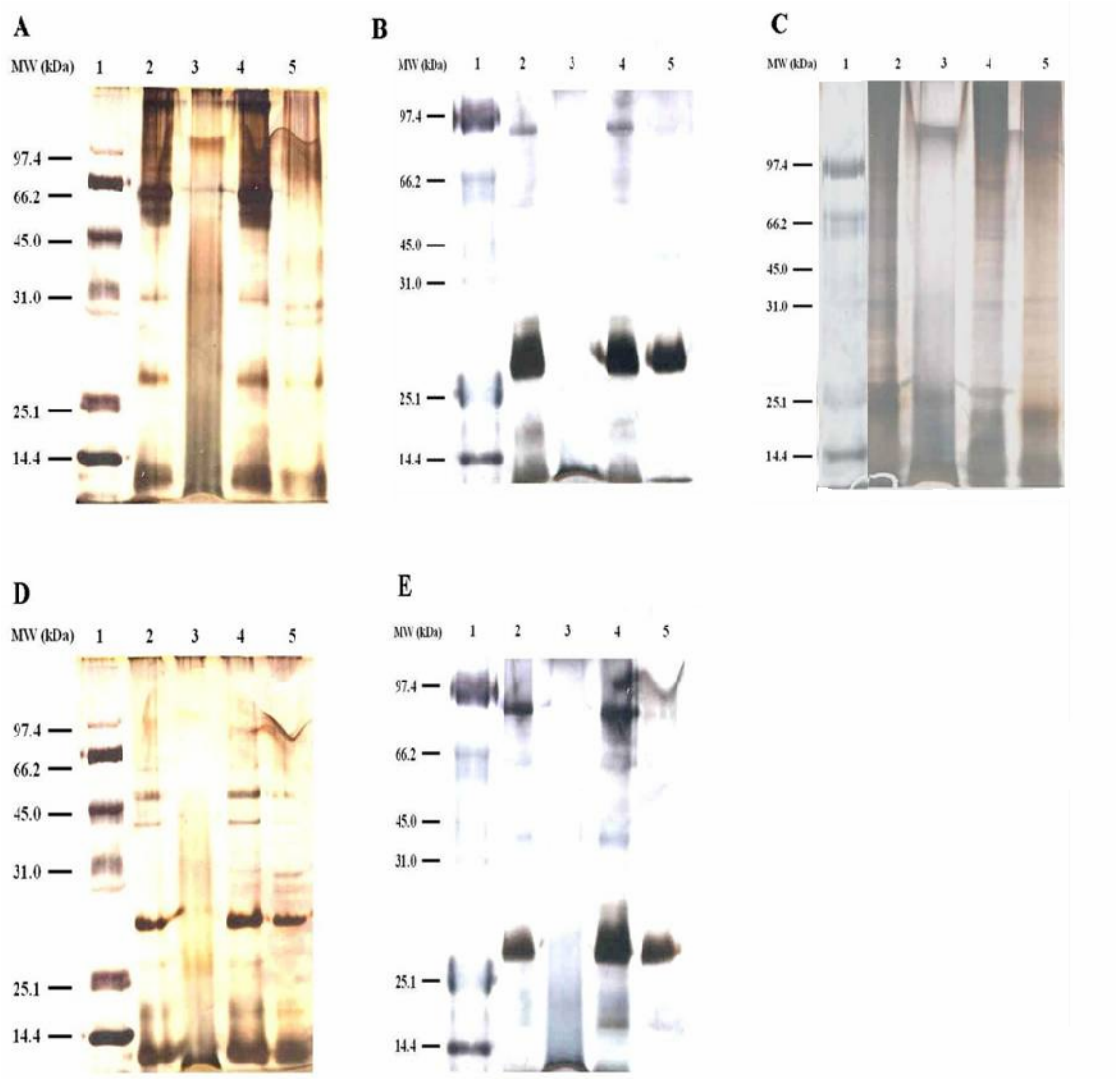


Figure 9 Silver staining SDS-PAGE of secreted antigen (SC) prepared from reference *L. interrogans* serovars Autumnalis (A), Bataviae (B), Javanica (C), Pyrogenes (D) and Sejroe (E). Molecular marker (MW) is shown on the left hand.

Lane 1: Molecular marker

Lane 2: Control Antigen for sodium periodate treatment

Lane 3: Antigen treated with sodium periodate

Lane 4: Control Antigen for proteinase K treatment

Lane 5: Antigen treated with proteinase K

2. Interaction of DC-SIGN with *L. interrogans*

Because leptospires contain high mannose glycans, which are ligands for DC-SIGN, as the major components on their cell surface. Therefore, it is interesting to evaluate the potential interaction of *L. interrogans* with DC-SIGN on dendritic cells. Leptospiral antigens were screened for their ability to bind DC-SIGN by ELISA using soluble DC-SIGN-Fc IgG as described in Materials and Methods (Soluble DC-SIGN-Fc Adhesion assay).

Brifely, whole cells and secreted antigens prepared from *L. interrogans* serovars Autumnalis, Bataviae, Javanica, Pyrogenes and Sejroe, which were the predominant serovar isolation in Thailand during year 2000 to 2004 [31], were coated onto polyvinyl plate at 37 ^{o}C for 2 h and blocked with skim milk. Soluble DC-SIGN-Fc IgG was then added into each well and followed by anti-human IgG- biotinylated conjugate, ST-AP and DNPP substrate. The substrate reaction was measured OD at 405 nm and the results were compared to controls. All *L. interrogans* either whole cells or secreted antigens bound to soluble DC-SIGN-Fc IgG (Figure 10, mean ± S.E., n=2). However, whole cell leptospires reacted to DC-SIGN stronger than the corresponding secreted antigens. Such a difference might be related to the concentration of antigens used in the plate coating. The concentration of protein in whole cell antigen was 2 times higher than the concentration of the secreted antigen.

The whole cells (WC) leptospires reacted to DC-SIGN about 1.6 to 1.8 folds higher than the corresponding secreted antigens (SC) for serovar Autumnalis (WC 0.713, SC 0.435), Bataviae (WC 0.815, SC 0.452) and Pyrogenes (WC 1.131, SC 0.676), whereas serovar Javanica (WC 0.701, SC 0.533) and serovar Sejroe (WC 0.889, SC 0.806) bound to soluble DC-SIGN-Fc IgG at similar levels.

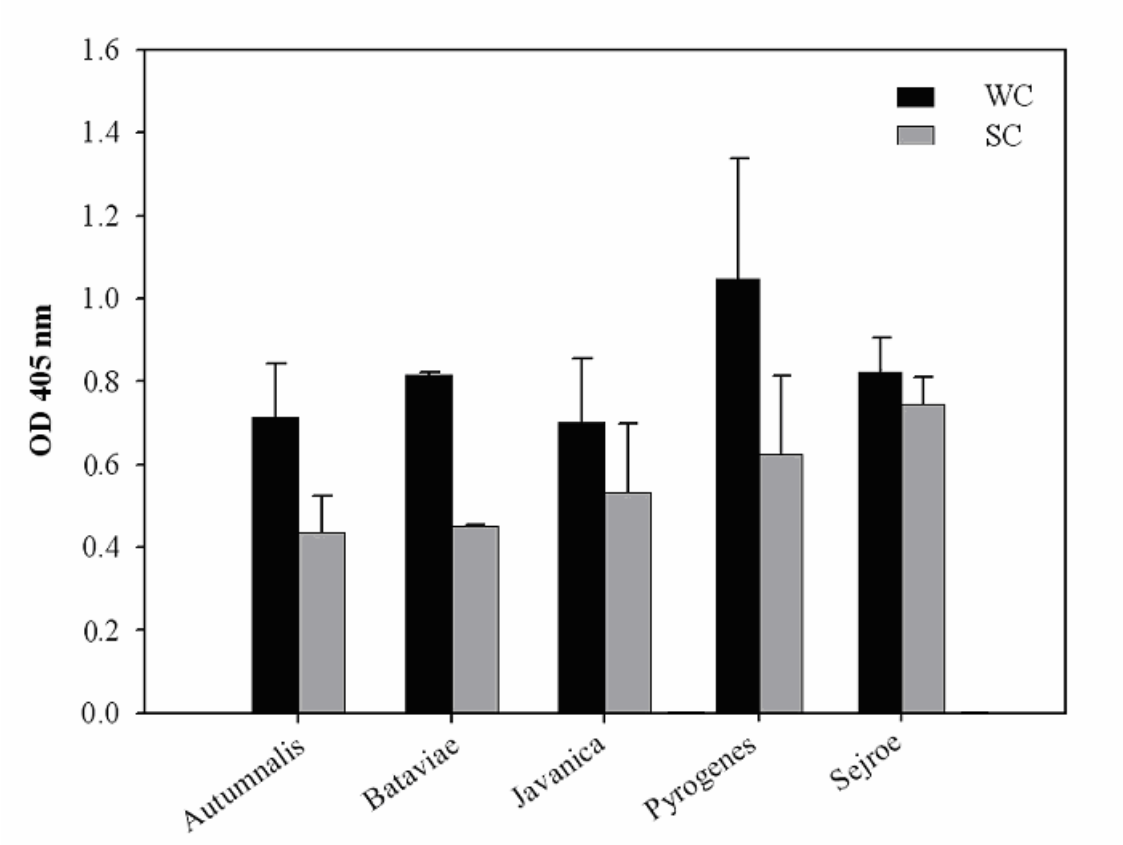


Figure 10 Binding of *L. interrogans* antigens both whole cell and secreted antigen using protein concentration of 10 μg/ml and 5 μg/ml, respectively to soluble DC-SIGN-Fc IgG**.** Whole cell (WC) and secreted antigen (SC) prepared from reference *L. interrogans* serovars Autumnalis, Bataviae, Javanica, Pyrogenes and Sejroe were coated onto each well of microtitter plate and determined their binding with soluble DC-SIGN-Fc IgG by ELISA. Data are shown as mean ± S.E., n = 2.

From these results, the whole cells leptospires were further investigated for specific binding to soluble DC-SIGN-Fc IgG by blocking experiment using mannan as DC-SIGN competitor. Mannan showed the highest effect on serovar Sejroe by reducing the percent binding about 8 folds (from 0.419±0.122 to 0.052±0.027) (Figure 11 A, mean ± S.E., n = 2). Serovar Autumnalis and Bataviae were reduced the binding about 7 folds (from 0.395±0.094 to 0.058±0.079 and from 0.563±0.107 to 0.083±0.122, respectively). The percent binding of serovar Javanica and Pyrogenes were reduced about 4.5 and 3 folds, respectively (from 0.366±0.036 to 0.082±0.113 and from 0.812±0.058 to 0.254±0.066).

In addition, *L. interrogans* serovar Pyrogenes 2317 virulent (vi) strain, its corresponding avirulent (avi) strain and clinical isolated *L. interrogans* serovars Autumnalis L-643 and BL-6 were used for detail analysis in further experiments. Indeed, all leptospires bound specifically to soluble DC-SIGN-Fc IgG, as indicated by abrogation of binding in the presence of mannan as shown in Figure 11 B (mean ± S.E., n = 2). Mannan showed the highest effect on serovar Pyrogenes 2317 avirulent strain as percent binding decreased about 52.6 folds (from 1.440 ±0.019 to 0.028±0.009) whereas virulent strain decreased the binding about 5.8 folds (from 1.207±0.107 to 0.207±0.013). Serovar Autumnalis strain L-643 was decreased the binding about 6.7 folds (from 1.268±0.034 to 0.190±0.029) whereas strain BL-6 was decreased the binding about 3.1 folds (from 0.332±0.026 to 0.104±0.011). These results suggested that *L. interrogans* serovars have different specific binding to soluble DC-SIGN-Fc IgG.

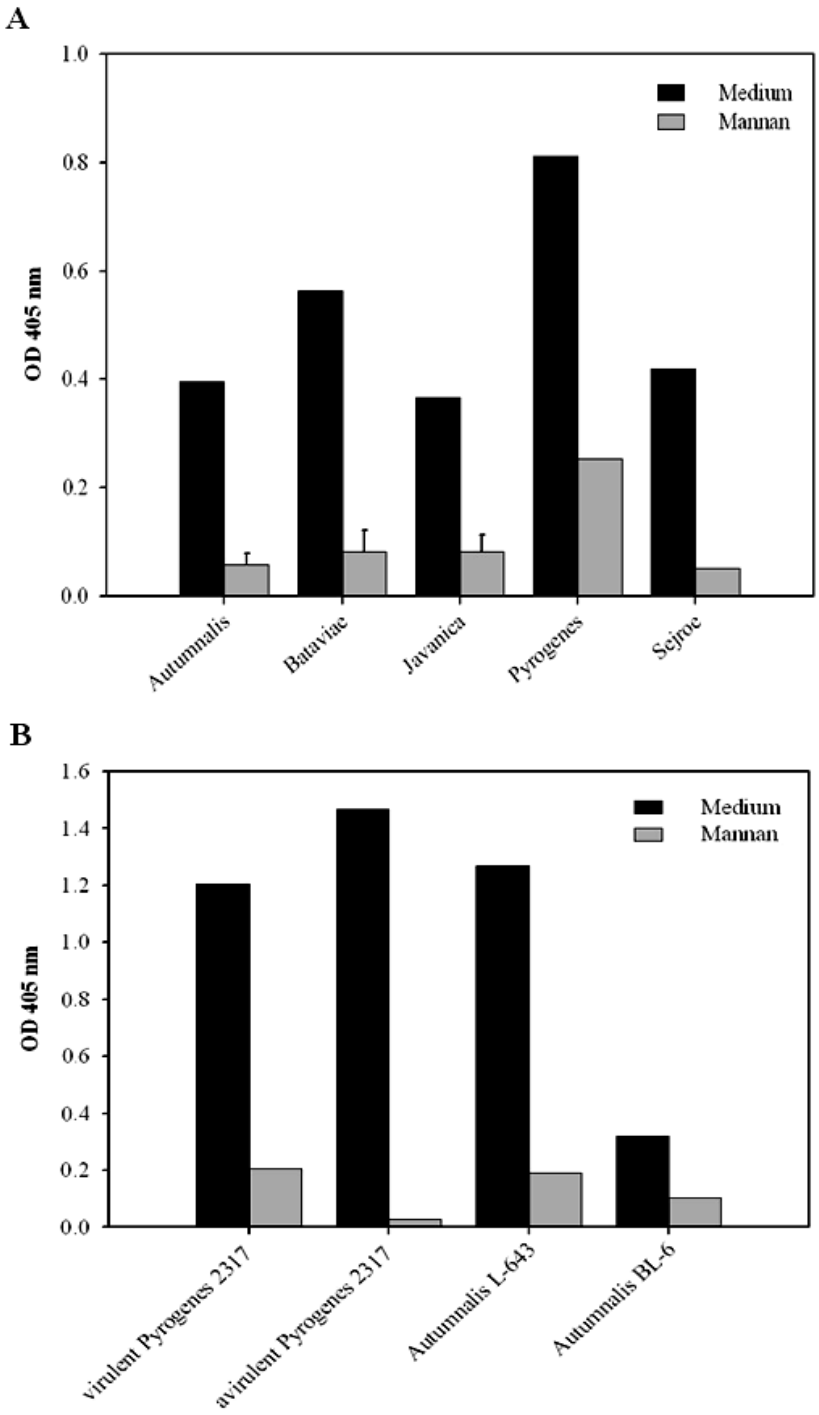


Figure 11 *L. interrogans* specifically bind to soluble DC-SIGN. Whole cell antigens prepared from the predominant serovars isolations in Thailand during year 2000 to 2004 (A) and clinical isolated *L. interrogans* (B), were analyzed for their specific binding to soluble DC-SIGN-Fc IgG by ELISA in the absence and presence of 50 μg/ml mannan. Data are shown as mean ± S.E., n = 2.

Then, live and paraformaldehyde killed *L. interrogans* were compared for their ability to bind to DC-SIGN. Virulent *L. interrogans* serovar Pyrogenes 2317 used as representative strain was coated onto wells of microtittre plate and the assay was performed as described above. The result showed comparable binding for both live and killed bacteria and also specificity in the presence of mannan as soluble DC-SIGN-Fc IgG competitor (Figure 12, mean ± S.E., n = 2). These results suggested that there were no difference in DC-SIGN binding in either live or paraformaldehyde killed leptospires.

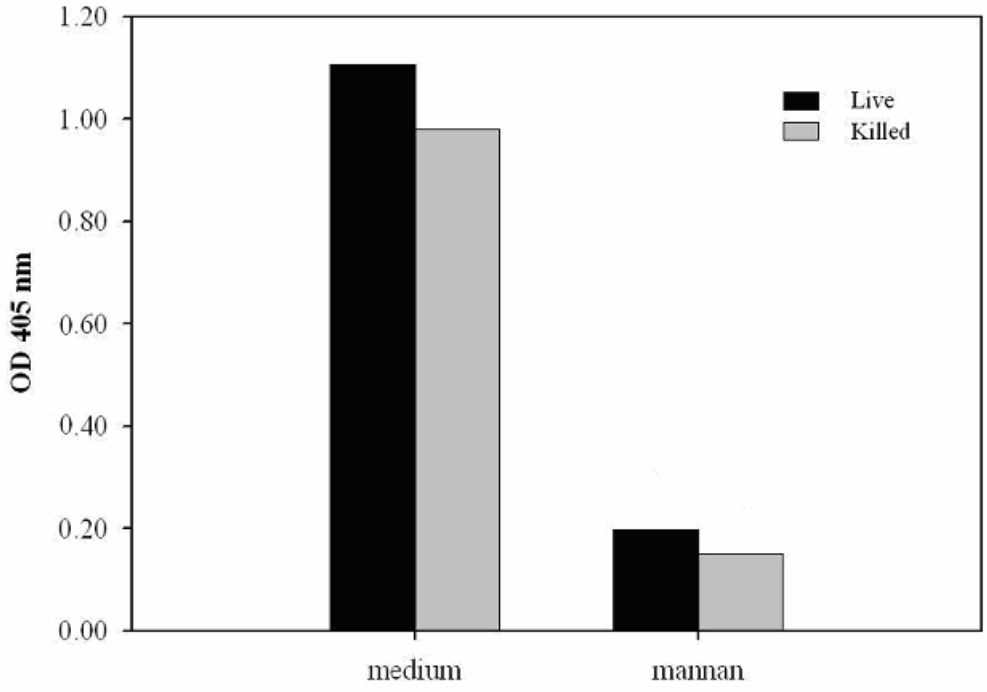


Figure 12 Live and paraformaldehyde-killed virulent *L. interrogans* serovar Pyrogenes 2317 were analyzed for their viability specific binding to soluble DC-SIGN-Fc IgG by ELISA in the presence of mannan 50 μg/ml (mean ± S.E., n = 2).

3. DC-SIGN expressing cells interacting with *L. interrogans*

To detect the interaction between DC-SIGN and *L. interrogans*, the assay was performed using K-562 cells transfected with DC-SIGN plasmid (K-SIGN). The stability of DC-SIGN expression on K-SIGN cells was tested by using monoclonal antibody specific to DC-SIGN coupled with FITC. K-562 cell was used as negative control. The results analyzed by flow cytometry demonstrated that K-SIGN cells expressed high level of DC-SIGN whereas K-562 cells did not express (Figure 13 A-B). MoDCs which derived from CD14+ cells in the present of GM-CSF and IL-4 were also analyzed for the DC-SIGN expression. The results were similar to the cell lines, K-SIGN (Figure 13 C).

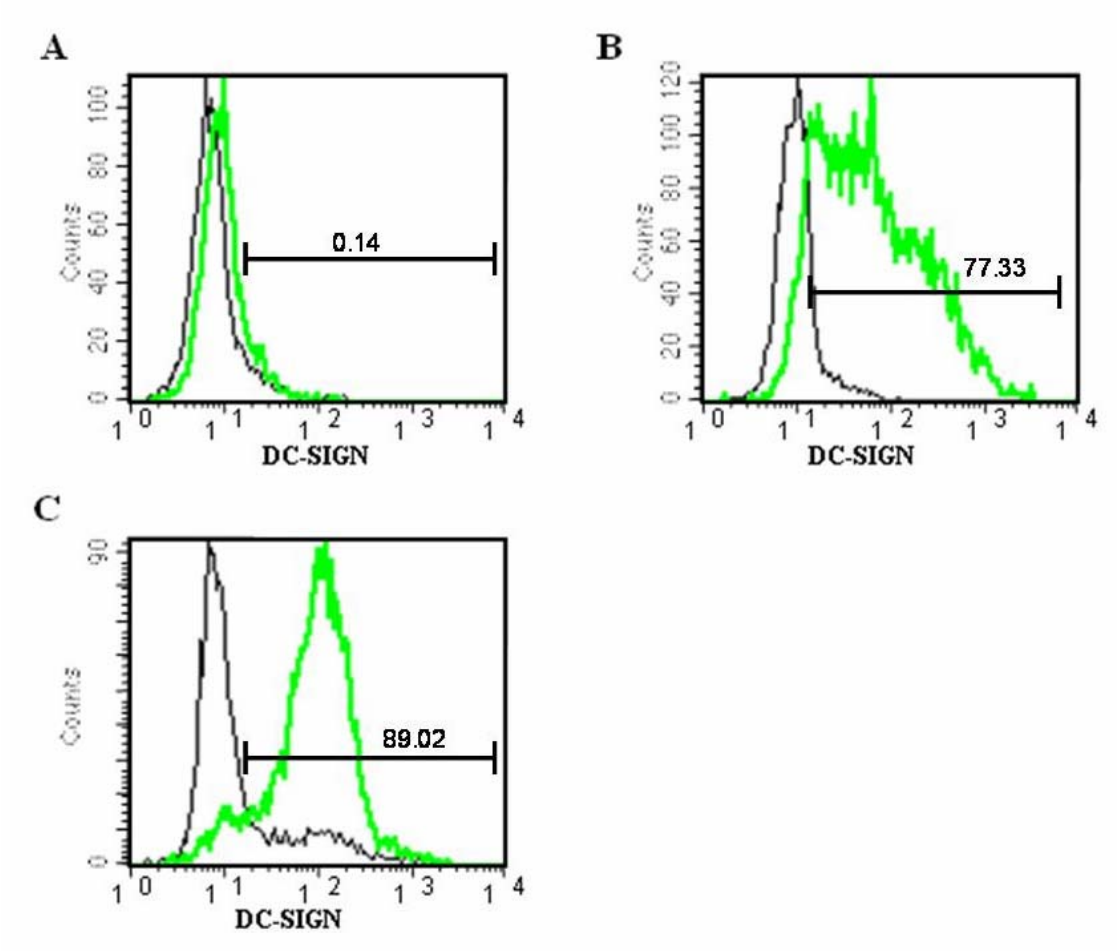


Figure 13 Histogram demonstrating the level of DC-SIGN expression on K-562 (A), K-562 transfected cells, K-SIGN (B) and MoDCs (C). The cells were stained with (gray) or without (black) FITC-conjugated anti-DC-SIGN (AZN-D1) antibody, and analyzed by flow cytometry. One representative data is shown.

The binding of leptospires to cellular DC-SIGN was performed by using fluorescent labeled bacteria and analyzed by flow cytometry. Labeled leptospires were added to K-562 and K-SIGN at ratio (cell:bacteria) of 1:10, 1:100 and 1:200 and the mixtures were incubated at 37 ^{o}C in CO_2 incubator for 40 min. The optimum binding ratio of 100 bacteria/cell which showed higher binding than ratio 1:10 and approximately as the binding at ratio 1:100, was chosen for further experiments (Figure 14). The leptospiral binding percentage was determined as percent of fluorescent detected cells of 10,000 cells in the gate of flow cytometry. As shown in Table 4 and 5 , Figure 15 (A-D) and 16 (mean ± S.E.), all *L. interrogans* serovars prefer binding to K-SIGN cells than K-562 cells in 4 independent experiments except avirulent Pyrogenes 2317 strains that has been tested for 2 independent experiments. In the presence of blocking reagents for DC-SIGN binding either mannan; EDTA or anti-DC-SIGN antibody (AZN-D1), the binding of all clinical isolated *L. interrogans* serovar Pyrogenes 2317 both virulent and avirulent strains and serovar Autumnalis L-643 and BL-6 strains were dramatically decreased when compared to the untreated samples. Interestingly, avirulent strain of serovar Pyrogenes 2317 showed more efficient on DC-SIGN binding than virulent strain (34.36±3.61 compared to 19.89±4.83). Moreover, this avirulent strain also showed more affectionate on DC-SIGN blocking than virulent strains in all blocking agents as 75 to 85% and 46 to 81% binding decreased of avirulent and virulent strains, respectively. Whereas these blocking agents showed less affect against serovar Autumnalis either L-643 or BL-6 strains which were decreased the binding 29 to 55% and 24 to 35%, respectively. Mannan and EDTA showed more affected on DC-SIGN binding than AZN-D1 for serovar Pyrogenes and Autumnalis clinical isolated. In deed, AZN-D1, anti-DE-SIGN antibody, showed more effective blocking to serovar Pyrogenes than serovar Autumnalis.

These results suggested that *L. interrogans* species bound to cellular DC-SIGN in different specific levels and the inhibitors, mannan, EDTA, AZN-D1, shown the most effect on DC-SIGN binding to serovar Pyrogenes.

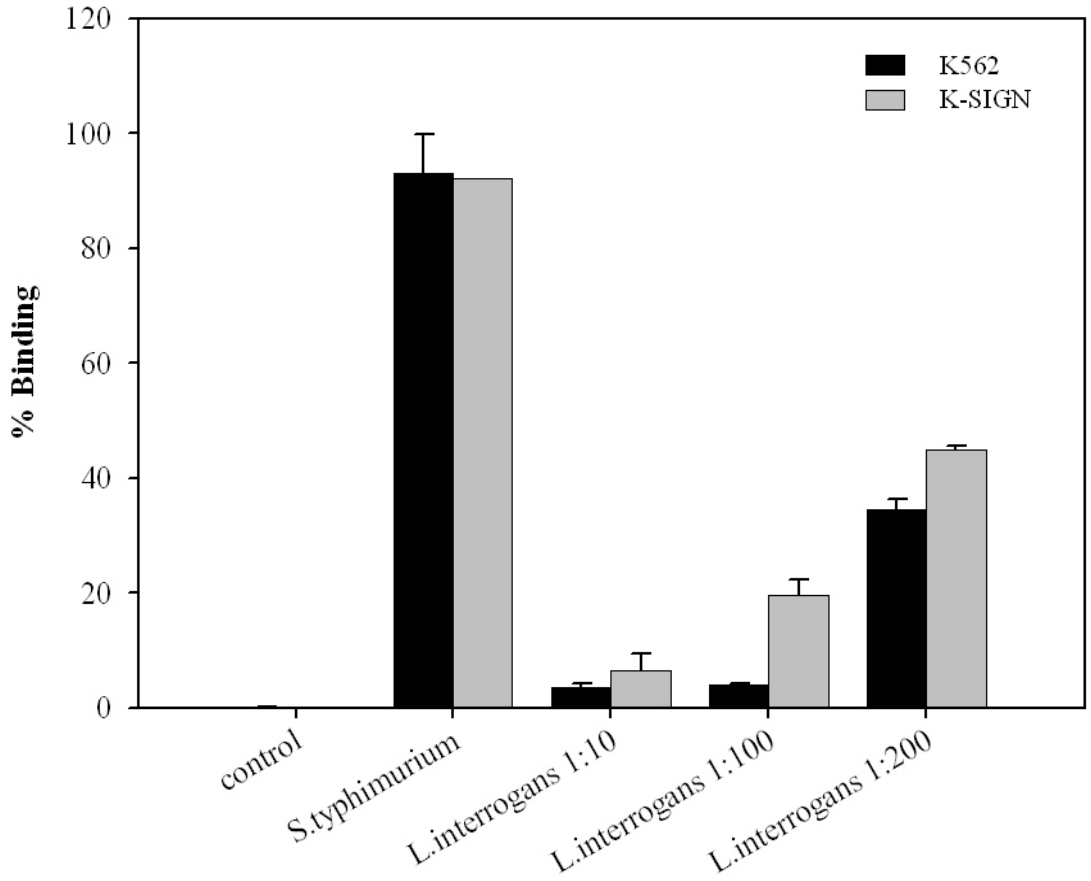


Figure 14. Binding of *L. interrogans* serovar Pyrogenes 2317 on K-562 and K-SIGN at different ratios (cell:bacteria 1:10, 1:100 and 1:200). *S. typhimurium* at cell:bacteria ratio 1:25 was used as positive control

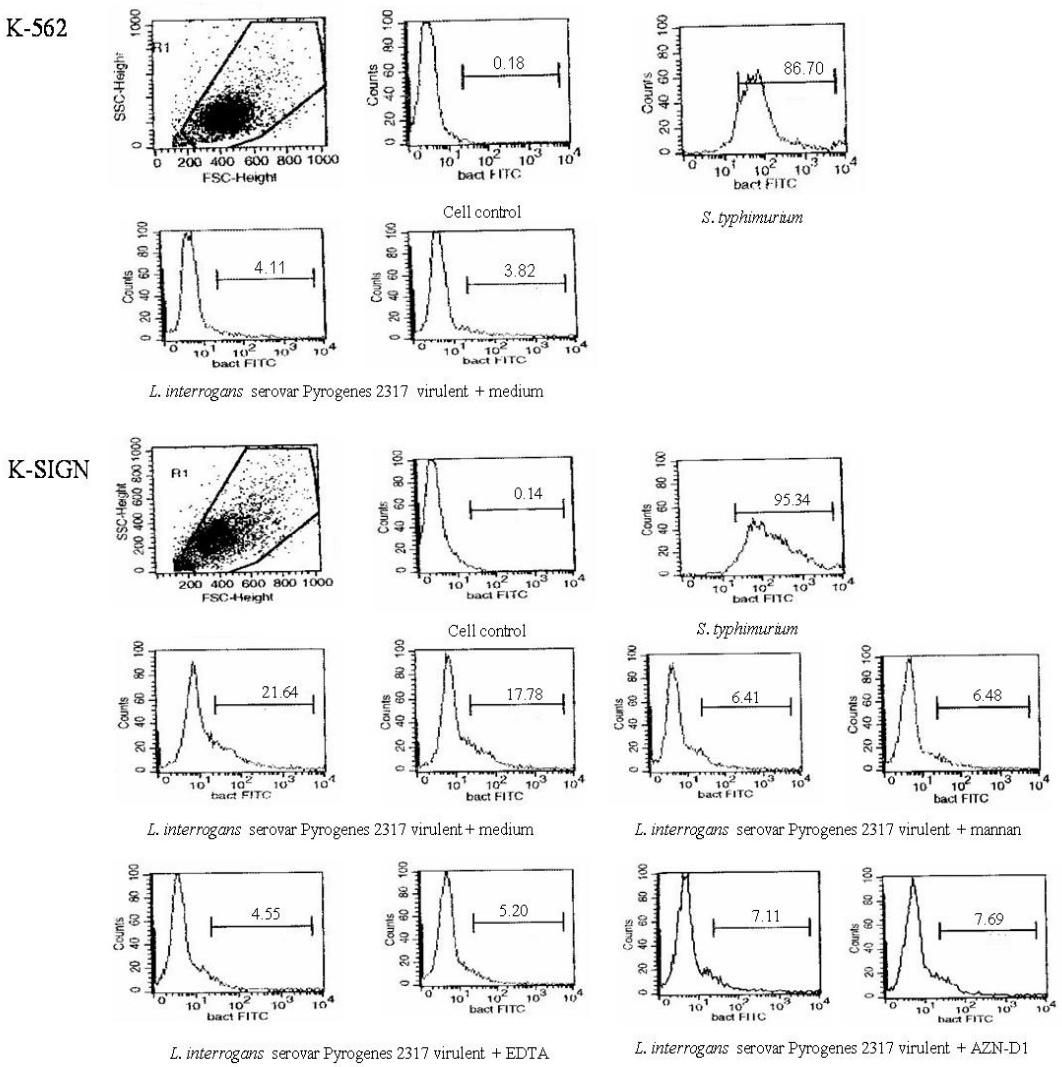
Table 4 Percent binding of *L. interrogans* serovar Pyrogenes 2317 virulent and avirulent strains to K-562 and K-SIGN in the presence and absence of DC-SIGN inhibitors which are mannan, EDTA, and anti-DC-SIGN (AZN-D1).

Experiment	Duplicate	Virulent					Avirulent				
		K-562	K-SIGN				K-562	K-SIGN			
			medium	mannan	EDTA	AZN-D1		medium	mannan	EDTA	AZN-D1
1	1	4.11	21.64	6.41	4.55	7.11	3.94	34.03	5.29	7.63	7.37
	2	3.82	17.78	6.48	5.20	7.69	5.06	29.40	5.36	7.20	7.59
2	1	8.42	22.60	8.92	1.20	6.23	12.52	36.50	5.41	4.19	7.46
	2	5.18	23.14	5.43	0.19	5.83	10.97	37.50	4.60	4.00	7.07
3	1	13.21	21.65	3.11	2.67	18.59	ND	ND	ND	ND	ND
	2	10.93	25.97	3.59	2.69	17.82	ND	ND	ND	ND	ND
4	1	1.58	11.24	5.14	7.69	10.38	ND	ND	ND	ND	ND
	2	2.13	15.10	6.46	6.43	12.71	ND	ND	ND	ND	ND
Mean		6.17	19.89	5.69	3.83	10.80	8.12	34.36	5.17	5.76	7.37
S.E.		4.23	4.83	1.84	2.59	5.11	4.26	3.61	0.38	1.93	0.22

Table 5 Percent binding of *L. interrogans* serovar Autumnalis L-643 and BL-6 strains to K-562 and K-SIGN in the presence and absence of DC-SIGN inhibitors which are mannan, EDTA, and anti-DC-SIGN (AZN-D1).

Experiment	Duplicate	L-643					BL-6				
		K-562	K-SIGN				K-562	K-SIGN			
			medium	mannan	EDTA	AZN-D1		medium	mannan	EDTA	AZN-D1
1	1	11.80	22.82	15.13	9.60	15.70	0.29	16.19	11.53	9.86	11.50
	2	13.69	23.07	15.20	9.19	16.10	0.28	12.03	10.66	10.71	9.25
2	1	11.63	28.17	21.37	14.09	19.53	6.52	11.47	9.12	12.34	9.22
	2	14.33	28.55	21.87	12.97	18.66	6.13	10.44	8.72	12.19	9.15
3	1	19.88	26.34	12.76	13.28	20.64	18.15	27.18	18.23	11.46	24.54
	2	23.90	23.99	11.13	13.19	17.45	17.86	32.92	18.73	18.43	16.320
4	1	15.55	23.13	9.02	8.21	16.74	6.00	26.95	16.51	17.71	24.41
	2	15.89	19.36	8.31	7.79	14.84	5.37	32.61	16.94	18.64	25.21
Mean		15.83	24.43	14.35	11.04	17.46	7.58	21.22	13.81	13.92	16.20
S.E.		4.18	3.09	5.14	2.58	2.01	6.91	9.68	4.21	3.69	7.44

A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FSC-Height
R1
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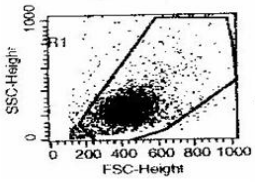
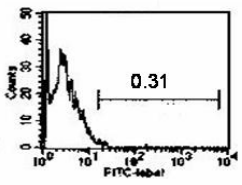
86.70
S. typhimurium
Cell control
4.11
3.82
L. interrogans serovar Pyrogenes 2317 virulent + medium
K-SIGN
0.14
95.34
S. typhimurium
Cell control
21.64
17.78
6.41
6.48
L. interrogans serovar Pyrogenes 2317 virulent + medium
L. interrogans serovar Pyrogenes 2317 virulent + mannan
4.55
5.20
7.11
7.69
L. interrogans serovar Pyrogenes 2317 virulent + EDTA
L. interrogans serovar Pyrogenes 2317 virulent + AZN-D1

B

K-562





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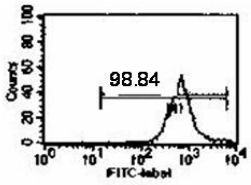
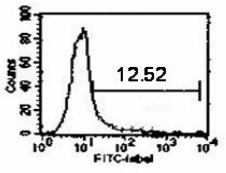
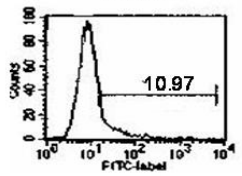


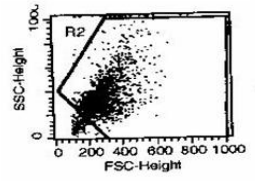
S. typhimurium

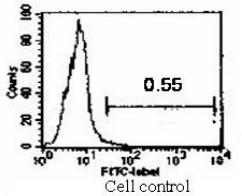




L. interrogans serovar Pyrogenes 2317 avirulent + medium

K-SIGN





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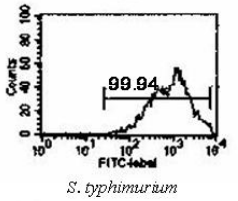
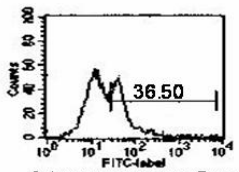
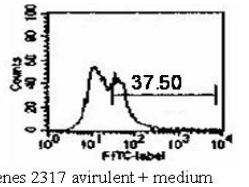
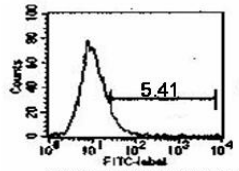


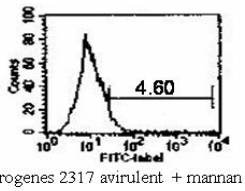
S. typhimurium



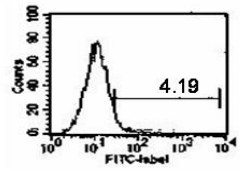


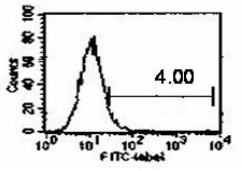
L. interrogans serovar Pyrogenes 2317 avirulent + medium



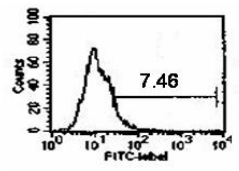


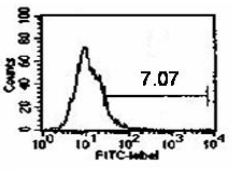
L. interrogans serovar Pyrogenes 2317 avirulent + mannan





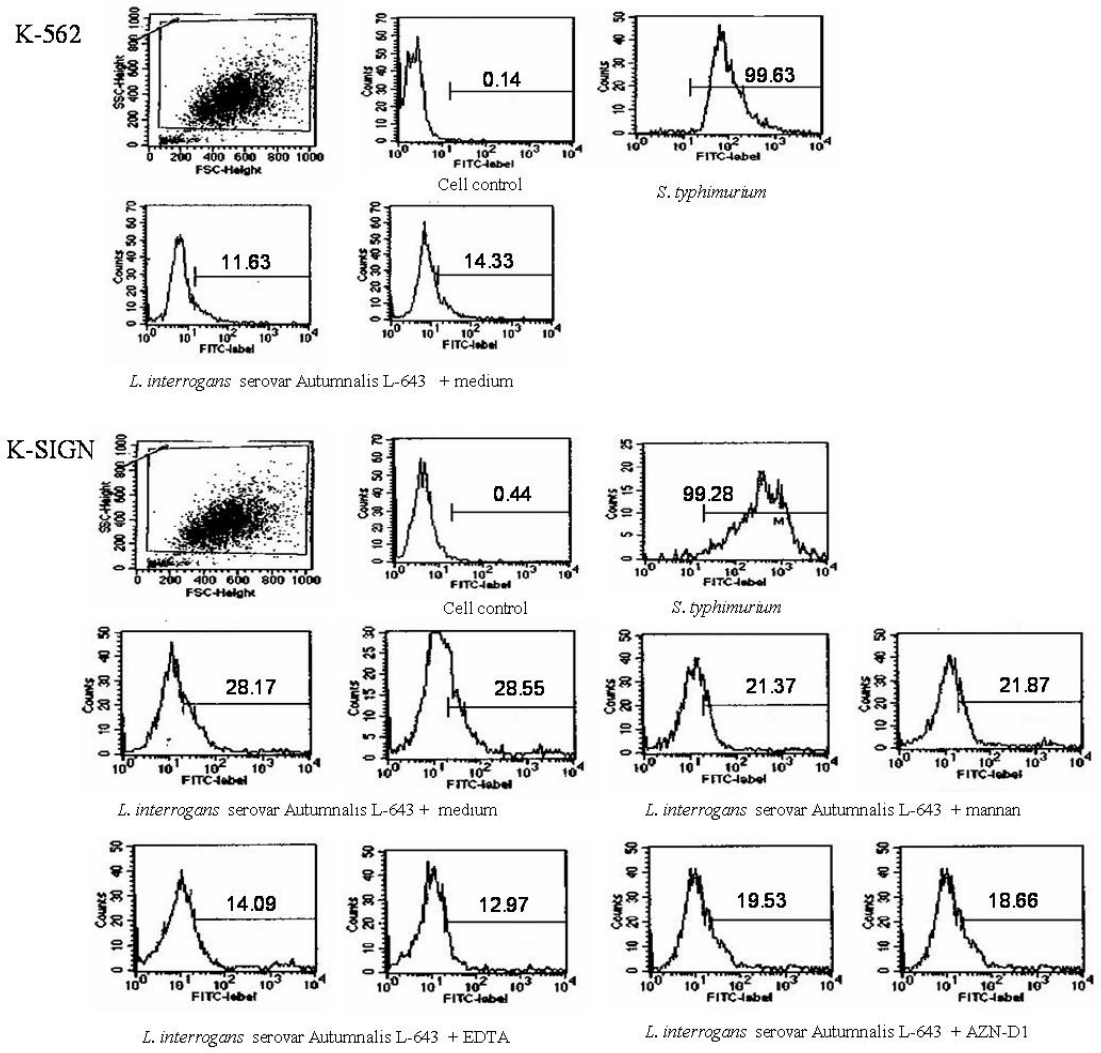
L. interrogans serovar Pyrogenes 2317 avirulent + EDTA





L. interrogans serovar Pyrogenes 2317 avirulent + AZN-D1

C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FSC-Height
0.14
Counts
FITC-label
Cell control
99.63
S. typhimurium
11.63
14.33
L. interrogans serovar Autumnalis L-643 + medium
K-SIGN
0.44
99.28
M
Cell control
S. typhimurium
28.17
28.55
21.37
21.87
L. interrogans serovar Autumnalis L-643 + medium
L. interrogans serovar Autumnalis L-643 + mannan
14.09
12.97
19.53
18.66
L. interrogans serovar Autumnalis L-643 + EDTA
L. interrogans serovar Autumnalis L-643 + AZN-D1

D

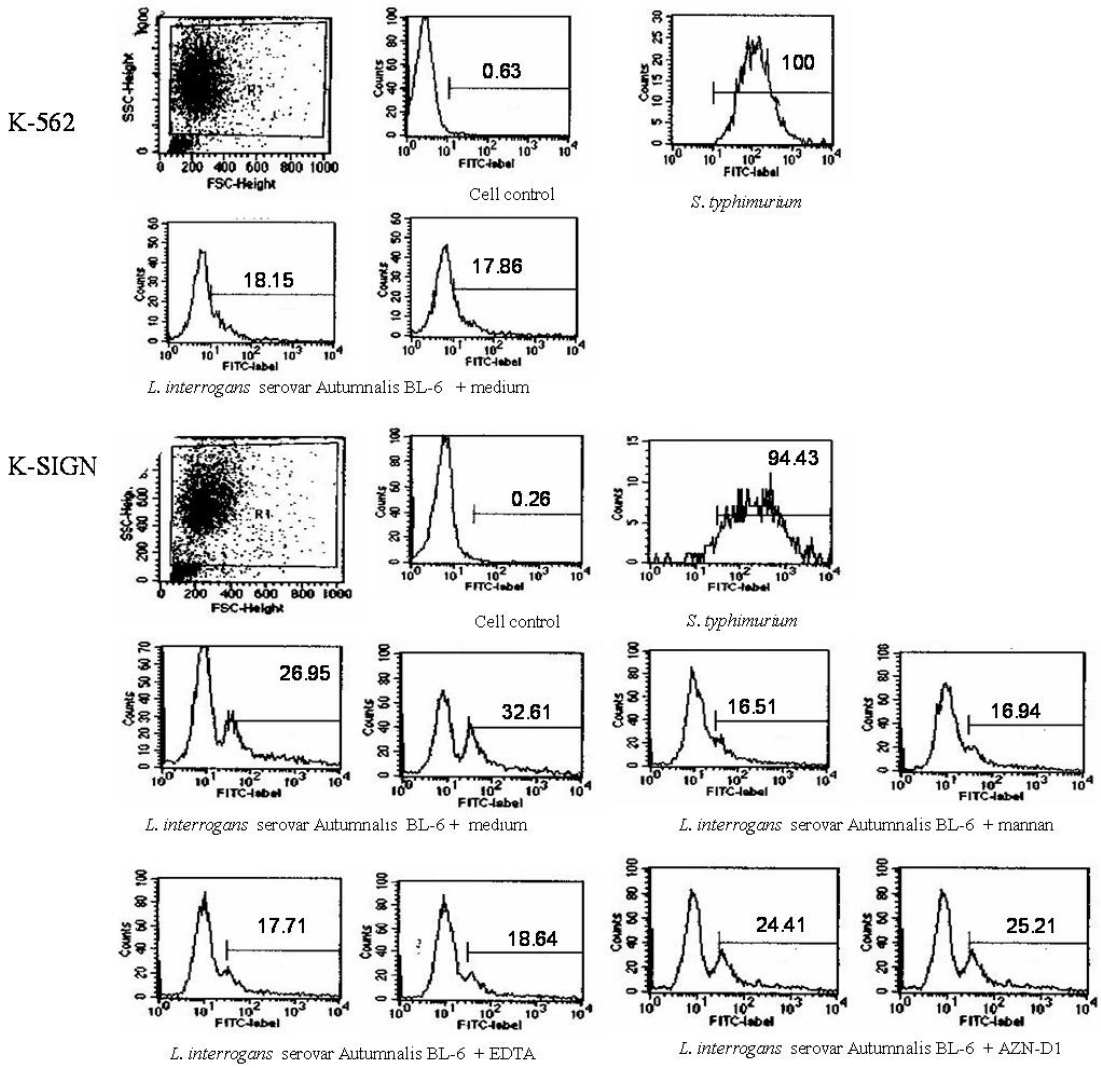


Figure 15. Histogram demonstrating the binding of *L. interrogans* serovar Pyrogenes 2317 virulent (A), avirulent (B), serovar Autumnalis L-643 (C) and BL-6 (D) strains to DC-SIGN on K-562, and K-SIGN. The results were analyzed by flow cytometry in the absence or presence of inhibitors which is mannan, EDTA, or anti-DC-SIGN (AZN-D1). One representative of 2 to 4 independent experiments are shown.

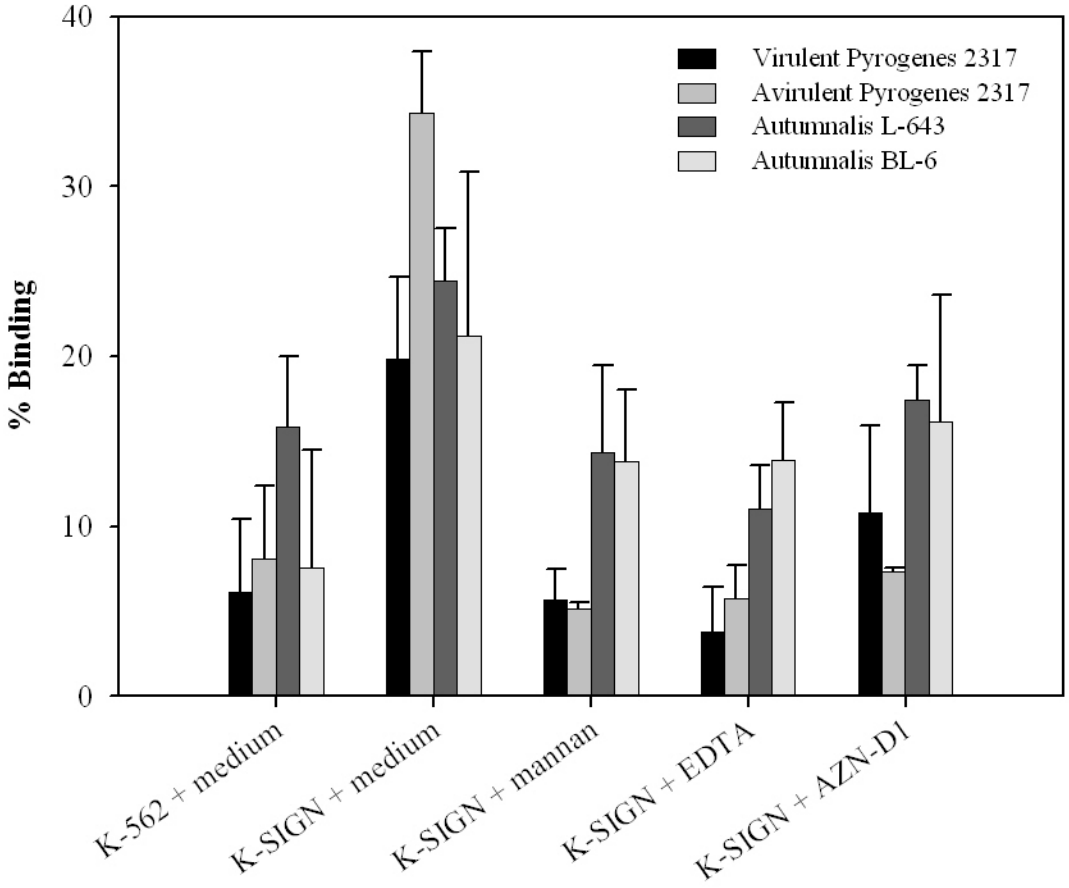


Figure 16 Percent binding of 4 leptospiral serovars to DC-SIGN expressing cells, K-SIGN. The cells were incubated with FITC-labeled *L. interrogans* at 37 ^{o}C for 40 minutes and then analyzed by flow cytometry. The specific interaction was tested by pre-incubating the cells with an inhibitor which is mannan, EDTA or anti-DC-SIGN (AZN-D1) before performing the experiment. Data are shown as mean±S.E. of *L. interrogans* serovar Pyrogenes 2317 virulent (n = 4), avirulent (n = 2), serovar Autumnalis L-643 (n = 4) and BL6 strains (n = 4).

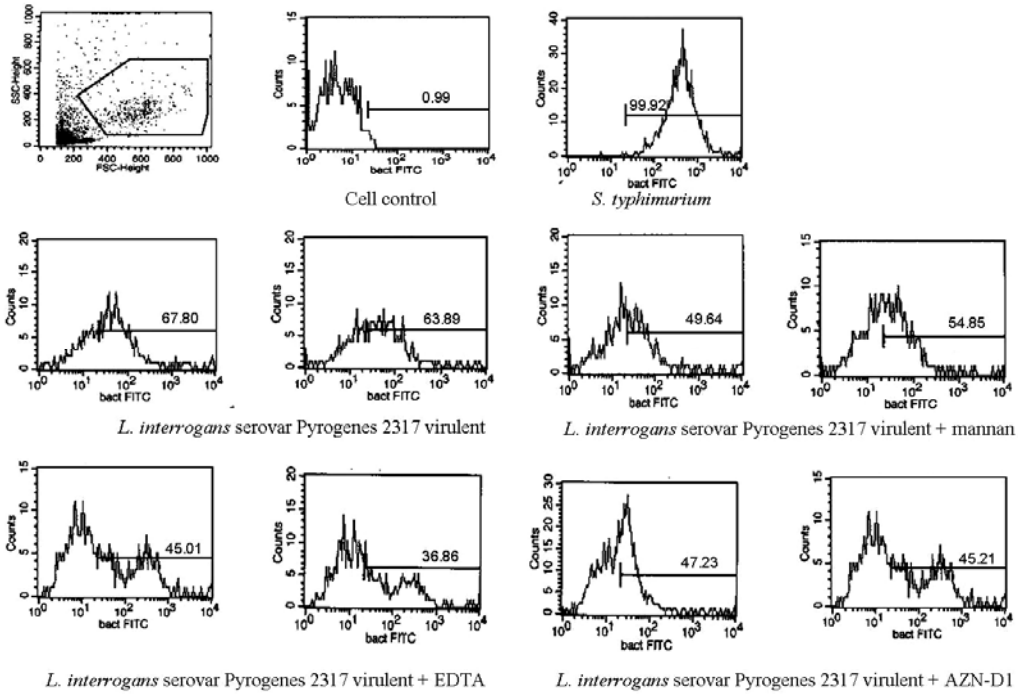
Next, DC-SIGN binding on DCs were further analyzed. There was much more efficiency of leptospires binding to DC-SIGN on MoDCs than on K-SIGN binding (Table 6 to 9 and Figure 17 to 20). The binding and blocking efficiency of *L. interrogans* were varied depend on MoDCs donors and one representative donor was shown in Figure 21. The binding of virulent *L. interrogans* serovar Pyrogenes 2317 were decreased in the present of EDTA (34 to 84%) more than AZN-D1 (29 to 71%) and mannan (12 to 43%) in all donors. Avirulent *L. interrogans* serovar Pyrogenes 2317 showed the most efficiency on binding when compared to the other serovars and the binding could be blocked by all inhibitors (23 to 48%). The binding of *L. interrogans* serovar Autumnalis L-643 strain was dramatically decreased in the present of inhibitors (67 to 91%), whereas the binding of serovar Autumnalis BL-6 strain was decreased by EDTA (30 to 89%), AZN-D1 (26 to 70%) and mannan (12 to 66%). Interestingly, *L. interrogans* serovar Autumnalis BL-6 which bound poorly to soluble DC-SIGN-Fc showed high binding on cell expressing DC-SIGN both K-SIGN and MoDCs. This strain might use the other receptor molecules cooperated in recognition in addition to DC-SIGN.

Thus, on DC-SIGN expressing cells either K-SIGN or MoDCs demonstrated that *L. interrogans* binding used DC-SIGN as one of a receptor and, at least, C-type lectin involved in the recognition of *L. interrognas*.

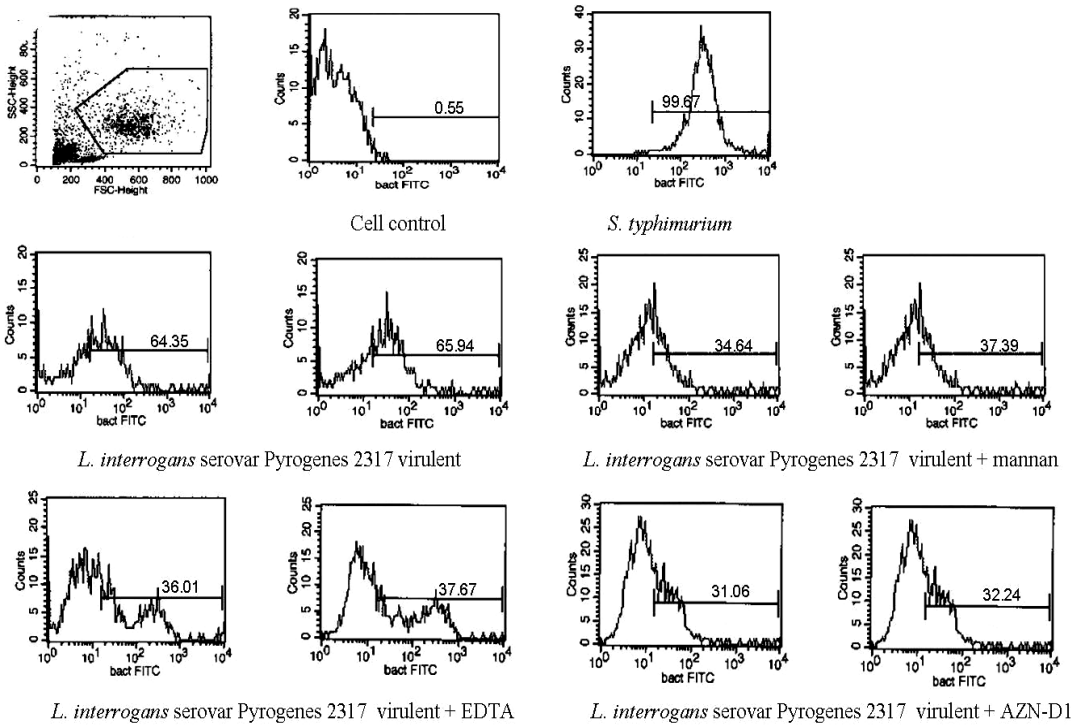
Table 6 Percent binding of *L. interrogans* serovar Pyrogenes 2317 (virulent strain) to MoDCs from 4 donors in the precent and absent of DC-SIGN inhibitors which are mannan, EDTA and anti-DC-SIGN (AZN-D1).
S. typhimurium and medium were used as positive and negative control, respectively.

Donor	**Duplicate**	**Control medium**	***S. typhimurium***	**Pyrogenes virulent strain 2317**			
				medium	**mannan**	**EDTA**	**AZN-D1**
D1	**1**	0.99	99.92	67.80	49.64	45.01	47.23
	2	ND	ND	63.89	54.85	36.86	45.21
D2	**1**	0.55	99.67	64.35	34.64	36.01	31.06
	2	ND	ND	65.94	37.39	37.67	32.24
D3	**1**	0.76	99.84	35.04	24.80	6.89	20.18
	2	ND	ND	31.03	27.26	4.92	18.57
D4	**1**	0.28	99.80	43.80	38.21	9.53	12.90
	2	ND	ND	41.63	24.47	8.37	13.11

A


SSC-Height
FSC-Height
R1
Counts
bact FITC
0.99
Cell control
99.92
S. typhimurium
67.80
63.89
L. interrogans serovar Pyrogenes 2317 virulent
49.64
54.85
L. interrogans serovar Pyrogenes 2317 virulent + mannan
45.01
36.86
L. interrogans serovar Pyrogenes 2317 virulent + EDTA
47.23
45.21
L. interrogans serovar Pyrogenes 2317 virulent + AZN-D1

B


SSC-Height
FSC-Height
Counts
bact FITC
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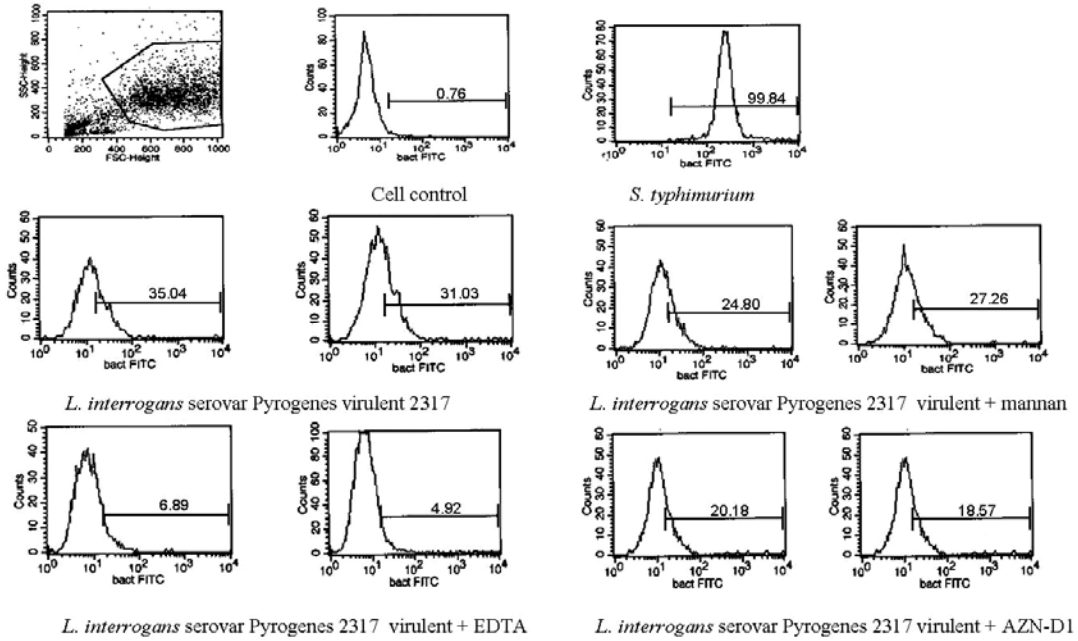
Cell control
99.67
S. typhimurium
64.35
65.94
L. interrogans serovar Pyrogenes 2317 virulent
34.64
37.39
L. interrogans serovar Pyrogenes 2317 virulent + mannan
36.01
37.67
L. interrogans serovar Pyrogenes 2317 virulent + EDTA
31.06
32.24
L. interrogans serovar Pyrogenes 2317 virulent + AZN-D1

C


SSC-Height
FSC-Height
Counts
bact FITC
0.76
Cell control
99.84
S. typhimurium
35.04
31.03
L. interrogans serovar Pyrogenes virulent 2317
24.80
27.26
L. interrogans serovar Pyrogenes 2317 virulent + mannan
6.89
4.92
L. interrogans serovar Pyrogenes 2317 virulent + EDTA
20.18
18.57
L. interrogans serovar Pyrogenes 2317 virulent + AZN-D1

D

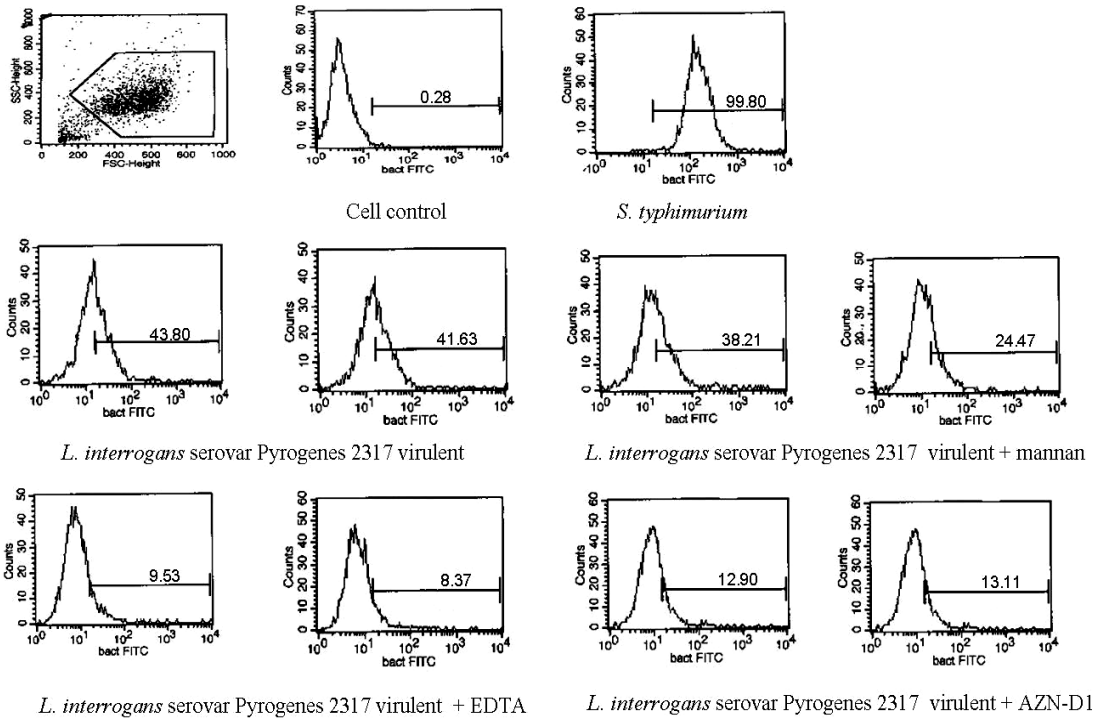
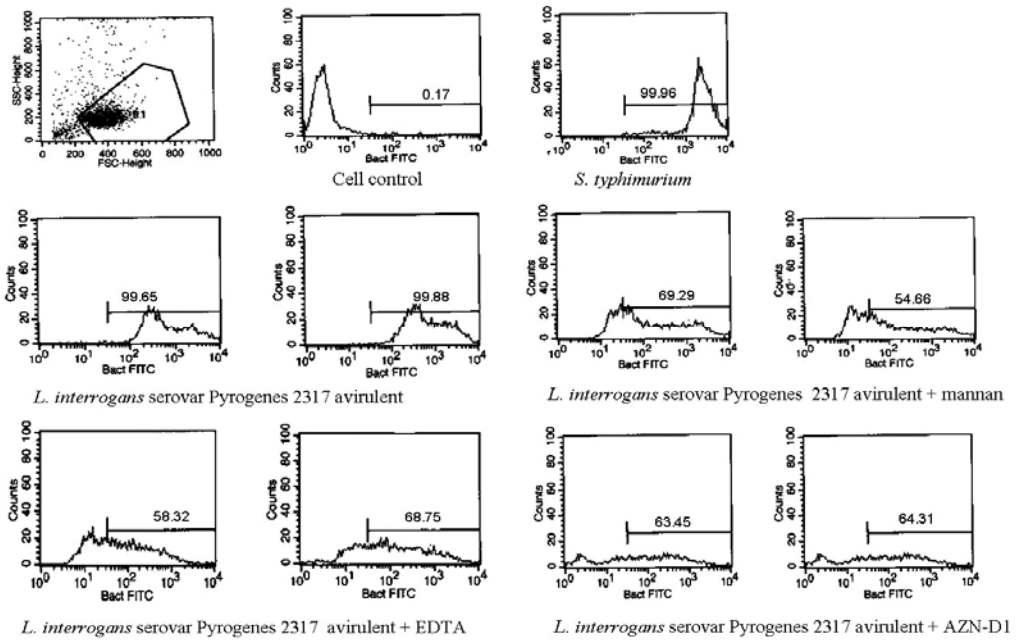


Figure 17. Histogram demonstrating the binding of *L. interrogans* serovar Pyrogenes 2317 virulent strain to MoDCs from individual donors (A-D). The specific interaction was tested by pre-incubating the cells with an inhibitor which is mannan, EDTA or anti-DC-SIGN (AZN-D1) before performing the experiment.

Table 7 Percent binding of *L. interrogans* serovar Pyrogenes 2317 (avirulent strain) to MoDCs from 2 donors in the precent and absent of DC-SIGN inhibitors which are mannan, EDTA and anti-DC-SIGN (AZN-D1).
S. typhimurium and medium were used as positive and negative control, respectively.

Donor	**Duplicate**	**Control medium**	***S. typhimurium***	**Pyrogenes avirulent strain 2317**			
				medium	**mannan**	**EDTA**	**AZN-D1**
D1	**1**	0.99	99.92	99.65	69.29	58.32	63.45
	2	ND	ND	99.88	54.68	68.75	64.31
D2	**1**	0.30	99.98	99.94	76.53	73.66	52.88
	2	ND	ND	99.98	74.58	72.38	51.82

A



B

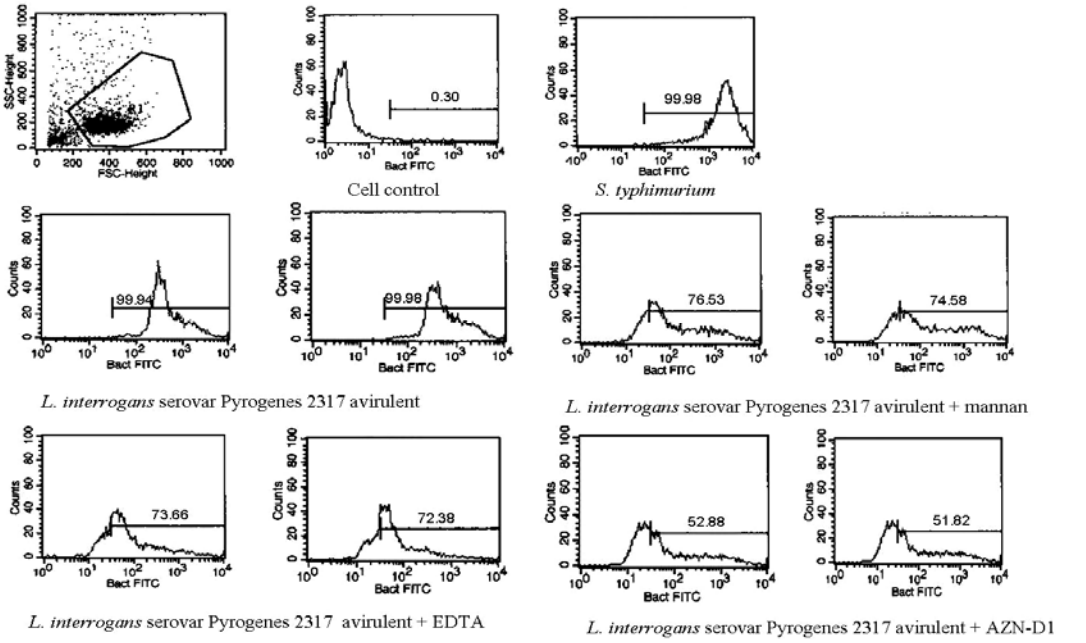
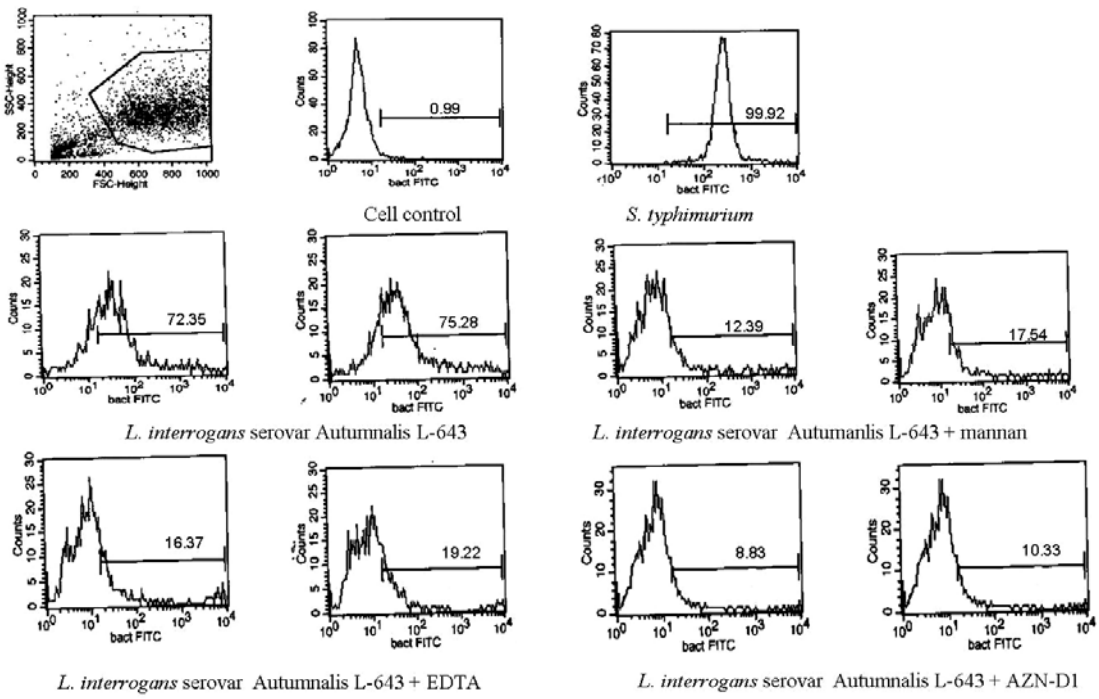


Figure 18. Histogram demonstrating the binding of *L. interrogans* serovar Pyrogenes 2317 avirulent strain to MoDCs from individual donors (A-B). The specific interaction was tested by pre-incubating the cells with an inhibitor which is mannan, EDTA or anti-DC-SIGN (AZN-D1) before performing the experiment.

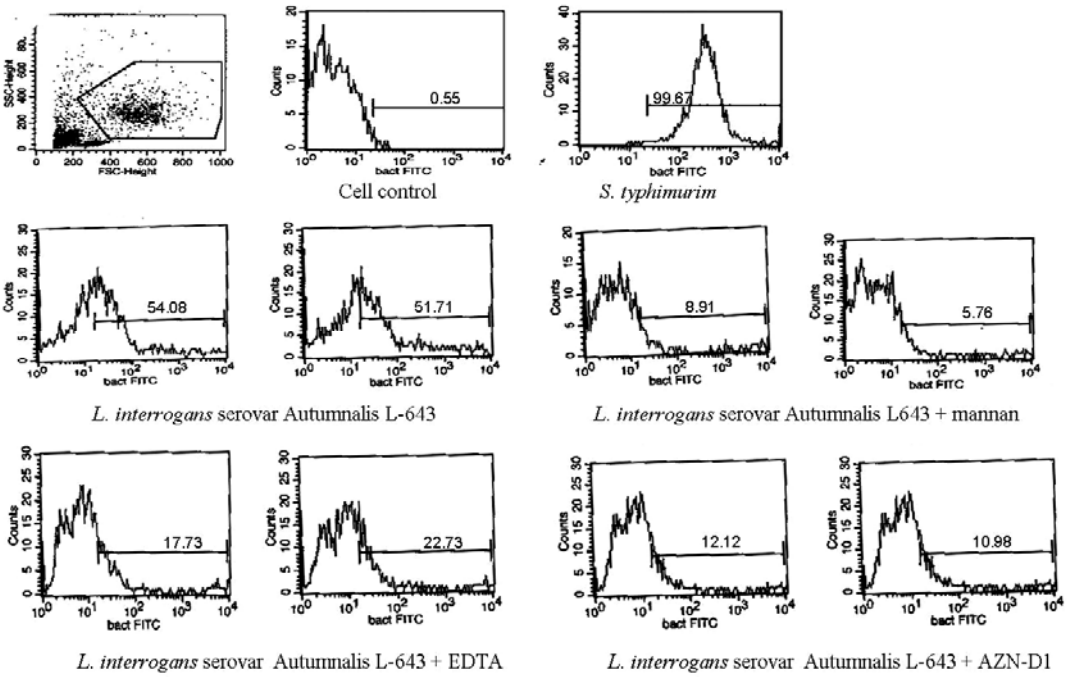
Table 8 Percent binding of *L. interrogans* serovar Autumnalis L-643 to MoDCs from 4 donors in the precent and absent of DC-SIGN inhibitors which are mannan, EDTA and anti-DC-SIGN (AZN-D1).
S. typhimurium and medium were used as positive and negative control, respectively.

Donor	**Duplicate**	**Control medium**	***S. typhimurium***	**L-643**			
				medium	**mannan**	**EDTA**	**AZN-D1**
D1	**1**	0.99	99.92	72.35	12.39	16.37	8.83
	2	ND	ND	75.28	17.54	19.22	10.33
D2	**1**	0.55	99.67	54.08	8.91	17.73	12.12
	2	ND	ND	51.17	5.76	22.73	10.98
D3	**1**	0.99	99.96	64.07	22.53	12.42	13.51
	2	ND	ND	54.11	8.51	9.28	11.82
D4	**1**	0.55	99.78	48.50	6.75	6.30	3.93
	2	ND	ND	49.35	5.28	9.72	5.54

A


SSC-Height
FSC-Height
Counts
bact FITC
0.99
Cell control
99.92
S. typhimurium
72.35
75.28
L. interrogans serovar Autumnalis L-643
12.39
17.54
L. interrogans serovar Autumnalis L-643 + mannan
16.37
19.22
L. interrogans serovar Autumnalis L-643 + EDTA
8.83
10.33
L. interrogans serovar Autumnalis L-643 + AZN-D1

B



Cell control

S. typhimurim

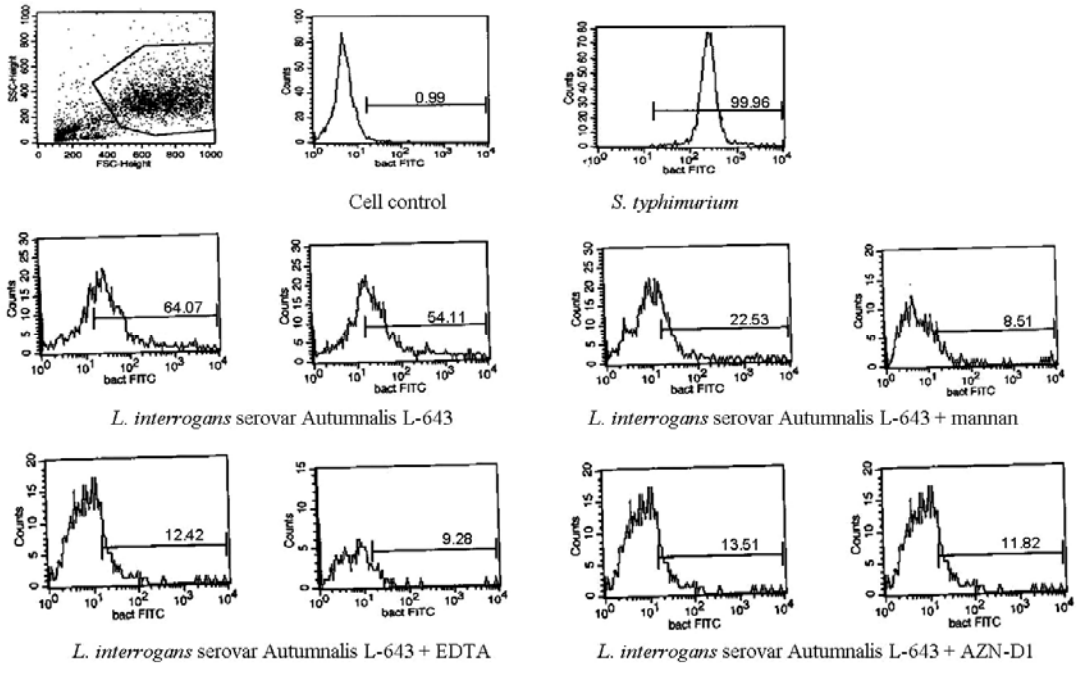
L. interrogans serovar Autumnalis L-643

L. interrogans serovar Autumnalis L643 + mannan

L. interrogans serovar Autumnalis L-643 + EDTA

L. interrogans serovar Autumnalis L-643 + AZN-D1

C



Cell control

S. typhimurium

L. interrogans serovar Autumnalis L-643

L. interrogans serovar Autumnalis L-643 + mannan

L. interrogans serovar Autumnalis L-643 + EDTA

L. interrogans serovar Autumnalis L-643 + AZN-D1

D

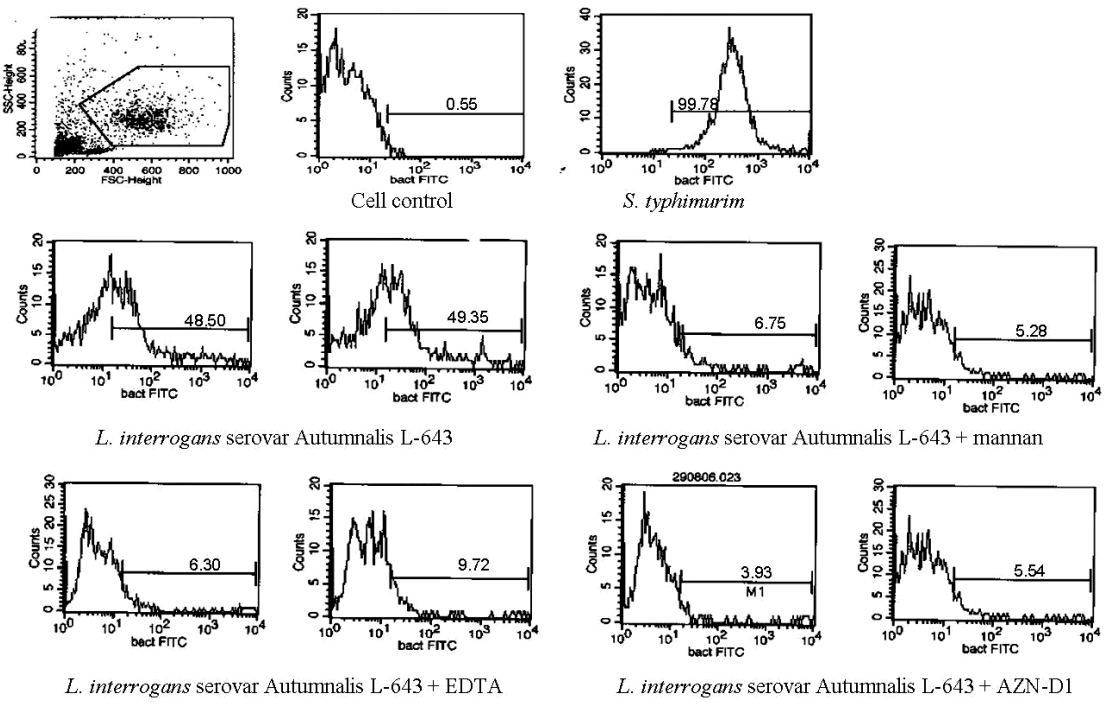
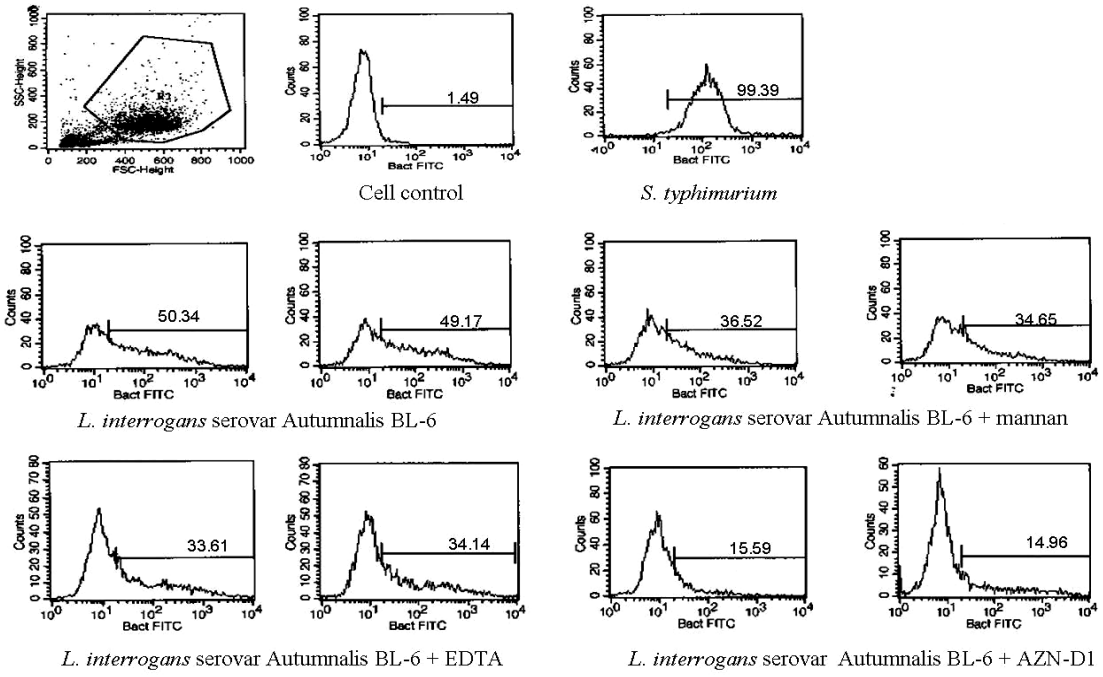


Figure 19. Histogram demonstrating the binding of *L. interrogans* serovar Autumnalis L-643 strain to MoDCs from individual donors (A-D). The specific interaction was tested by pre-incubating the cells with an inhibitor which is mannan, EDTA or anti-DC-SIGN (AZN-D1) before performing the experiment.

Table 9 Percent binding of *L. interrogans* serovar Autumnalis BL-6 to MoDCs from 4 donors in the precent and absent of DC-SIGN inhibitors which are mannan, EDTA and anti-DC-SIGN (AZN-D1).
S. typhimurium and medium were used as positive and negative control, respectively.

Donor	**Duplicate**	**Control medium**	***S. typhimurium***	**BL-6**			
				medium	**mannan**	**EDTA**	**AZN-D1**
D1	**1**	0.99	99.92	50.34	36.52	33.61	15.59
	2	ND	ND	49.17	34.65	34.14	14.96
D2	**1**	1.67	94.34	61.32	29.13	38.78	32.10
	2	ND	ND	58.75	24.61	32.32	30.33
D3	**1**	1.89	99.22	81.57	31.42	23.98	29.20
	2	ND	ND	80.57	27.49	22.71	30.16
D4	**1**	1.02	97.16	80.23	70.38	10.73	59.65
	2	ND	ND	80.00	67.57	8.58	58.25

A



Cell control

S. typhimurium

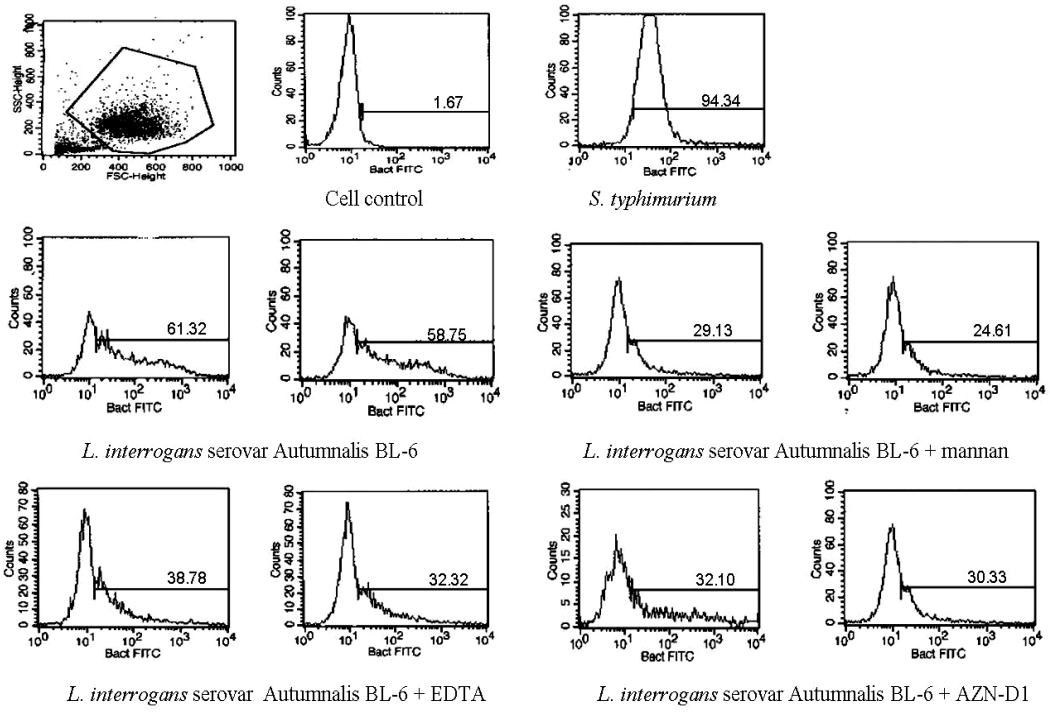
L. interrogans serovar Autumnalis BL-6

L. interrogans serovar Autumnalis BL-6 + mannan

L. interrogans serovar Autumnalis BL-6 + EDTA

L. interrogans serovar Autumnalis BL-6 + AZN-D1

B



Cell control

S. typhimurium

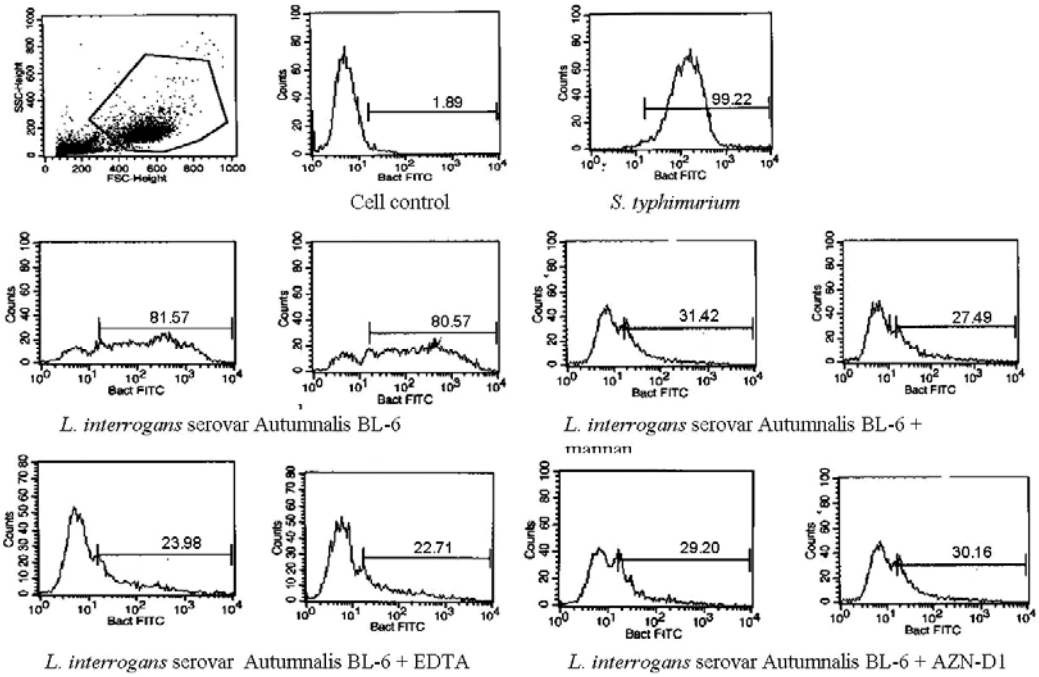
L. interrogans serovar Autumnalis BL-6

L. interrogans serovar Autumnalis BL-6 + mannan

L. interrogans serovar Autumnalis BL-6 + EDTA

L. interrogans serovar Autumnalis BL-6 + AZN-D1

C



Cell control

S. typhimurium

L. interrogans serovar Autumnalis BL-6

L. interrogans serovar Autumnalis BL-6 + mannan

L. interrogans serovar Autumnalis BL-6 + EDTA

L. interrogans serovar Autumnalis BL-6 + AZN-D1

D

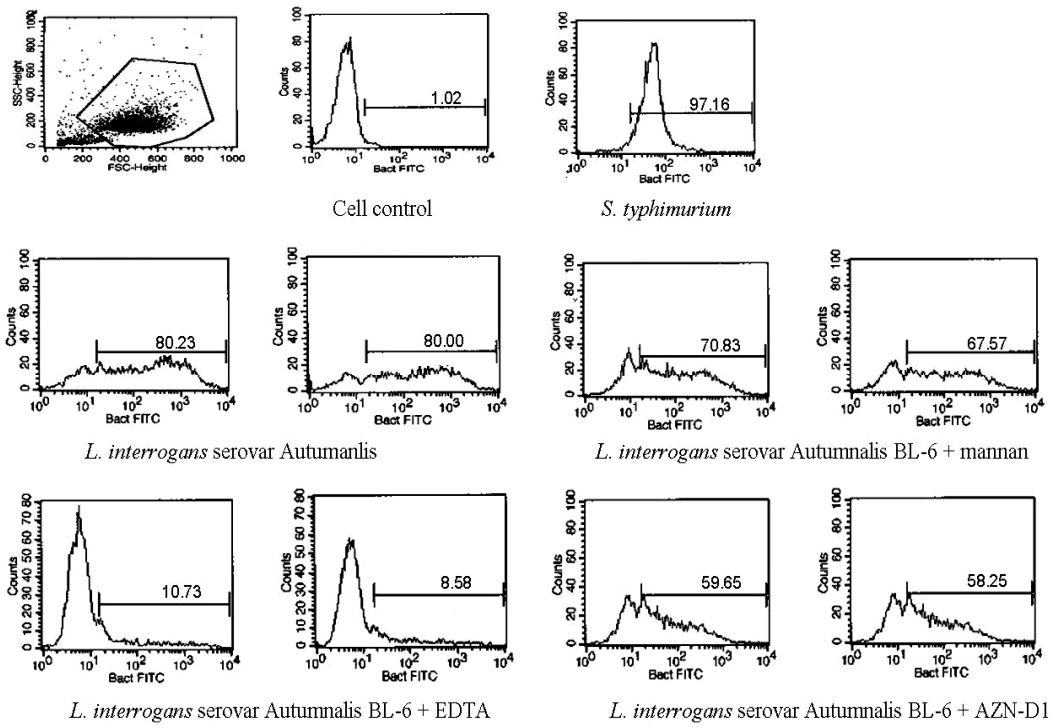


Figure 20. Histogram demonstrating the binding of *L. interrogans* serovar Autumnalis BL-6 strain to MoDCs from individual donors (A-D). The specific interaction was tested by pre-incubating the cells with an inhibitor which is mannan, EDTA or anti-DC-SIGN (AZN-D1) before performing the experiment.

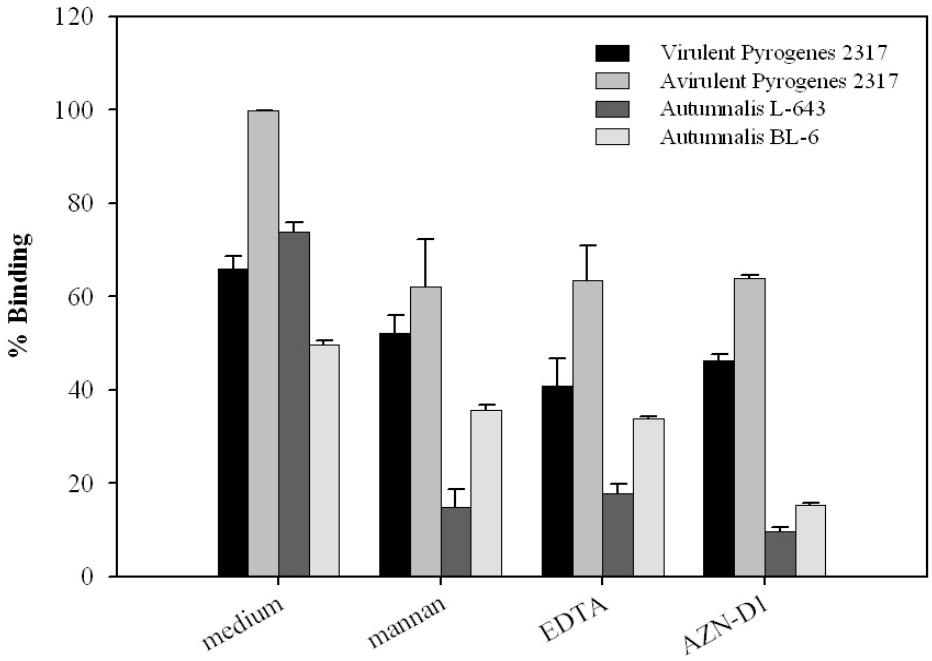
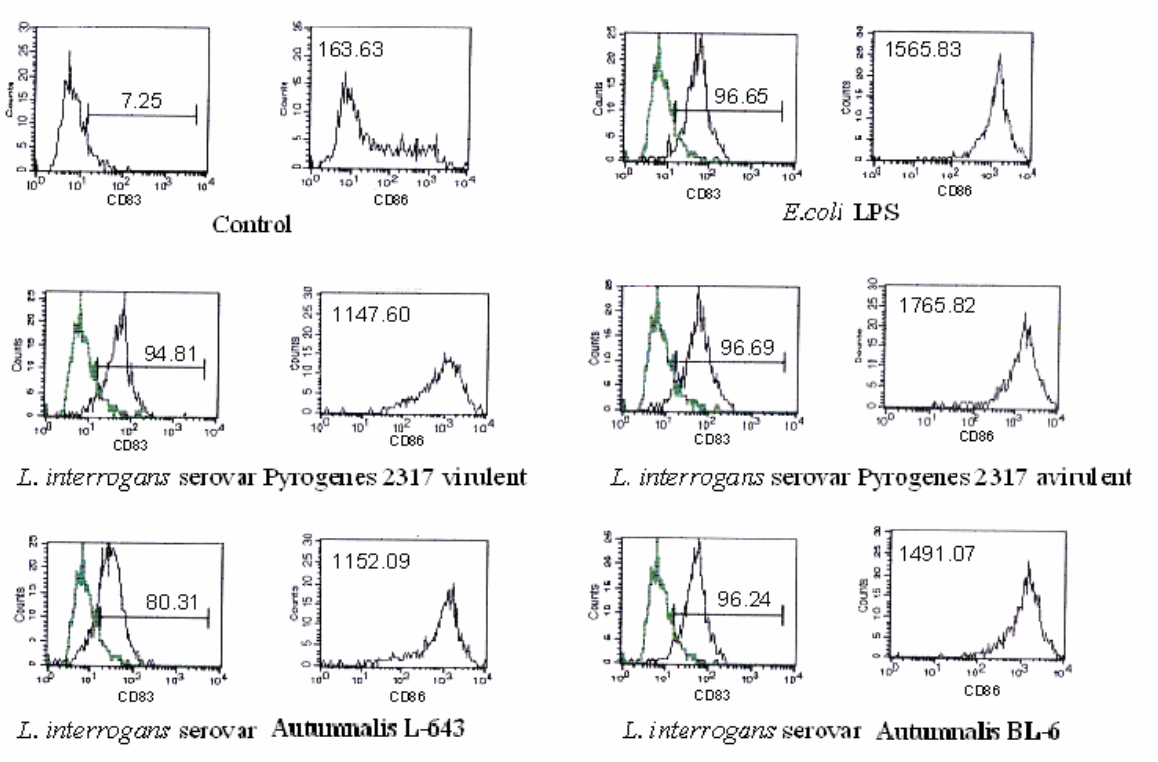


Figure 21 Percent binding of 4 leptospiral serovars to DC-SIGN on MoDCs. The cells were incubated with FITC-labeled *L. interrogans* at 37 ^{o}C for 40 minutes and then analyzed by flow cytometry. The specific interaction was tested by pre-incubating the cells with an inhibitor which is mannan, EDTA or anti-DC-SIGN (AZN-D1) before performing the experiment. Data from 1 of 4 representative donors is shown as mean±S.E..

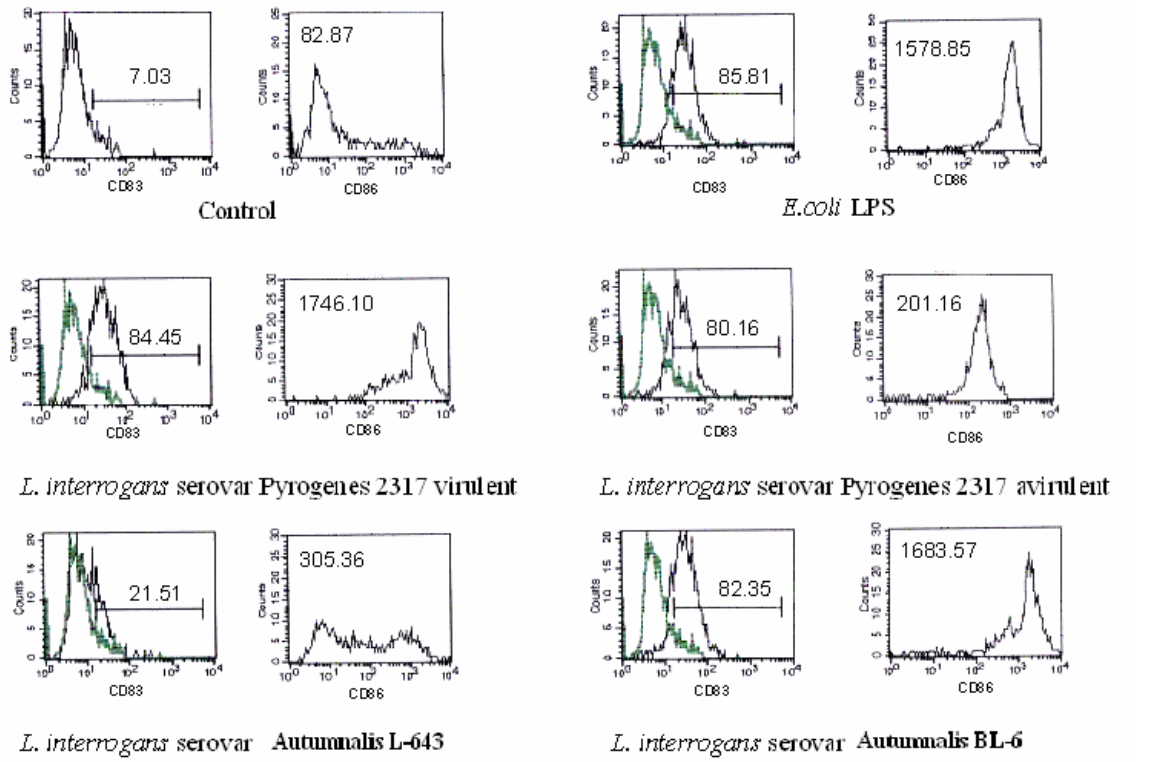
4. DCs maturation induced by *L. interrogans*

The influence of *L. interrogans* binding on DC maturation and cytokine production was investigated. MoDCs were cocultured with *L. interrogans* for 2 days; subsequently, the cells were stained with the labeled antibodies specific to the DC maturation marker (CD83) and costimulatory molecules (CD86) and the supernatant was tested for secretion of bioactive IL-12p70, IL-10 and TNF-α by ELISA. *E. coli* LPS was used as a positive control. As shown in Figure 22 (A-E), all *L. interrogans* induced DC maturation judging from increased expression of CD83 and CD86. All *L. interrogans* strains induced full DC maturation with exception of serovar Autumnalis L-643 that induced partially DC maturation in 1 of 5 donors.

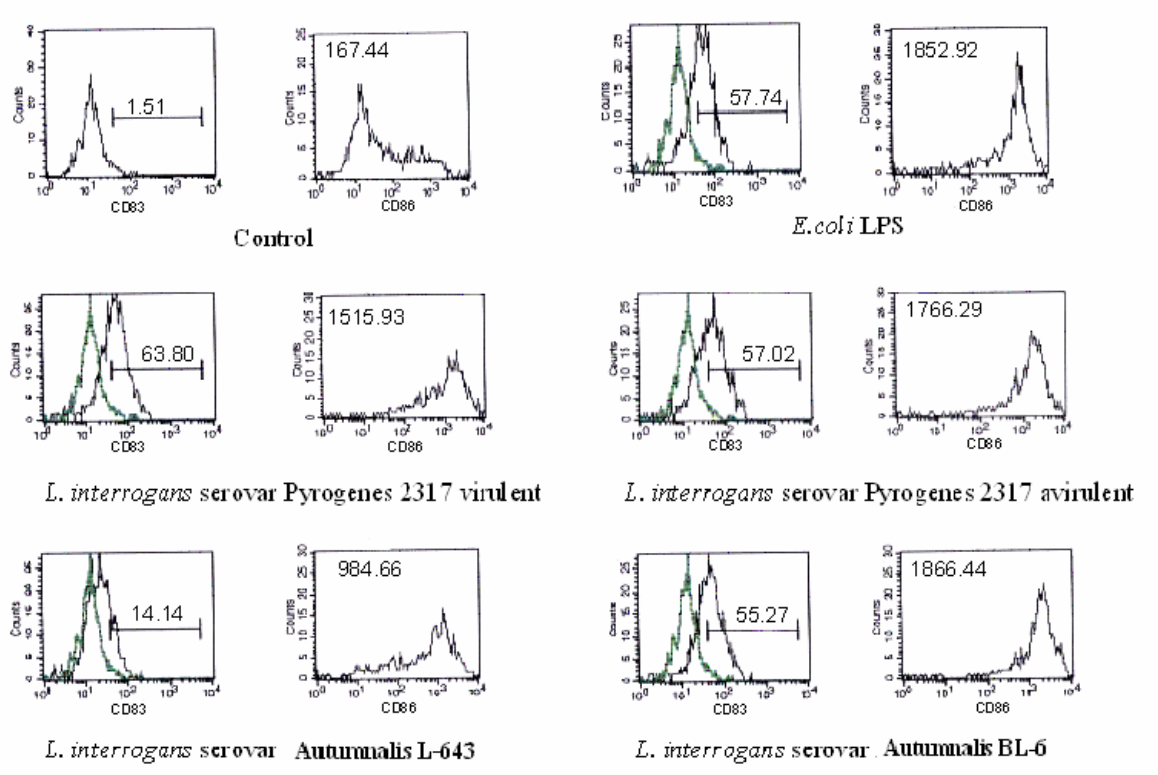
A


7.25
163.63
CD83
CD86
Control
96.65
1565.83
E.coli LPS
94.81
1147.60
L. interrogans serovar Pyrogenes 2317 virulent
96.69
1765.82
L. interrogans serovar Pyrogenes 2317 avirulent
80.31
1152.09
L. interrogans serovar Autumnalis L-643
96.24
1491.07
L. interrogans serovar Autumnalis BL-6

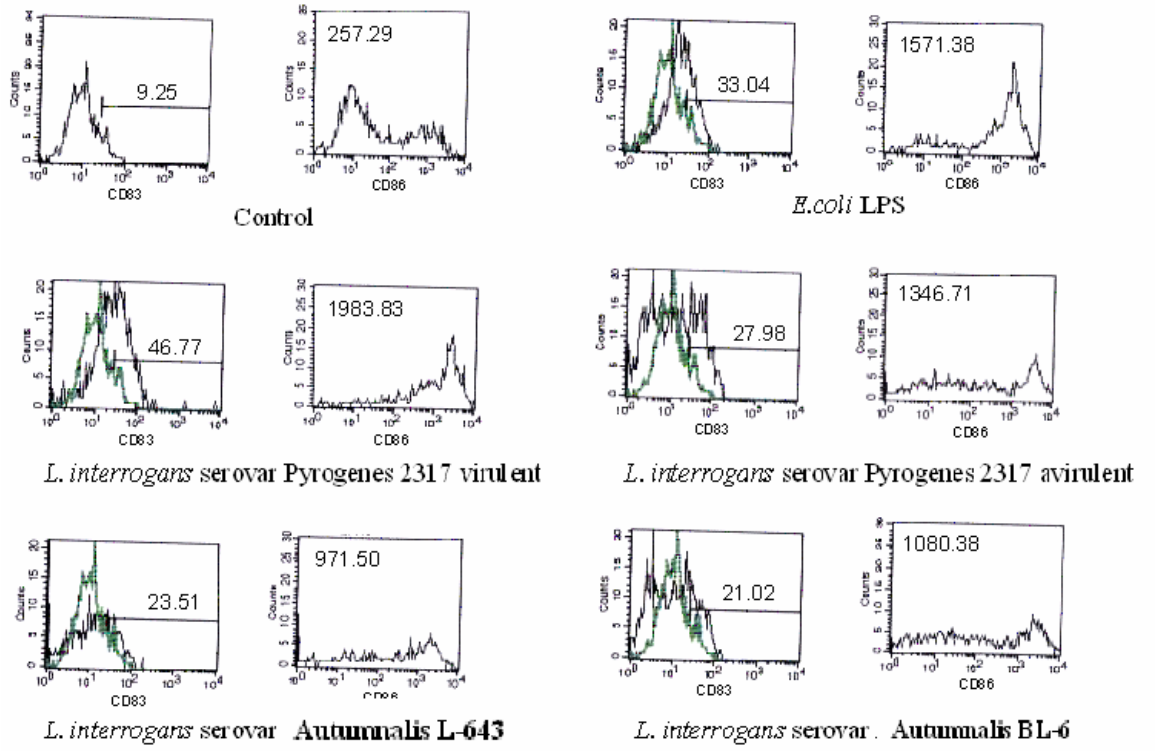
B


7.03
82.87
CD83
CD86
Control
85.81
1578.85
E.coli LPS
84.45
1746.10
L. interrogans serovar Pyrogenes 2317 virulent
80.16
201.16
L. interrogans serovar Pyrogenes 2317 avirulent
21.51
305.36
L. interrogans serovar Autumnalis L-643
82.35
1683.57
L. interrogans serovar Autumnalis BL-6

C


1.51
167.44
Control
57.74
1852.92
E.coli LPS
63.80
1515.93
L. interrogans serovar Pyrogenes 2317 virulent
57.02
1766.29
L. interrogans serovar Pyrogenes 2317 avirulent
14.14
984.66
L. interrogans serovar Autumnalis L-643
55.27
1866.44
L. interrogans serovar Autumnalis BL-6
CD83
CD86
Counts

D


9.25
257.29
Control
33.04
1571.38
E.coli LPS
46.77
1983.83
L. interrogans serovar Pyrogenes 2317 virulent
27.98
1346.71
L. interrogans serovar Pyrogenes 2317 avirulent
23.51
971.50
L. interrogans serovar Autumnalis L-643
21.02
1080.38
L. interrogans serovar Autumnalis BL-6
CD83
CD86
Counts

E

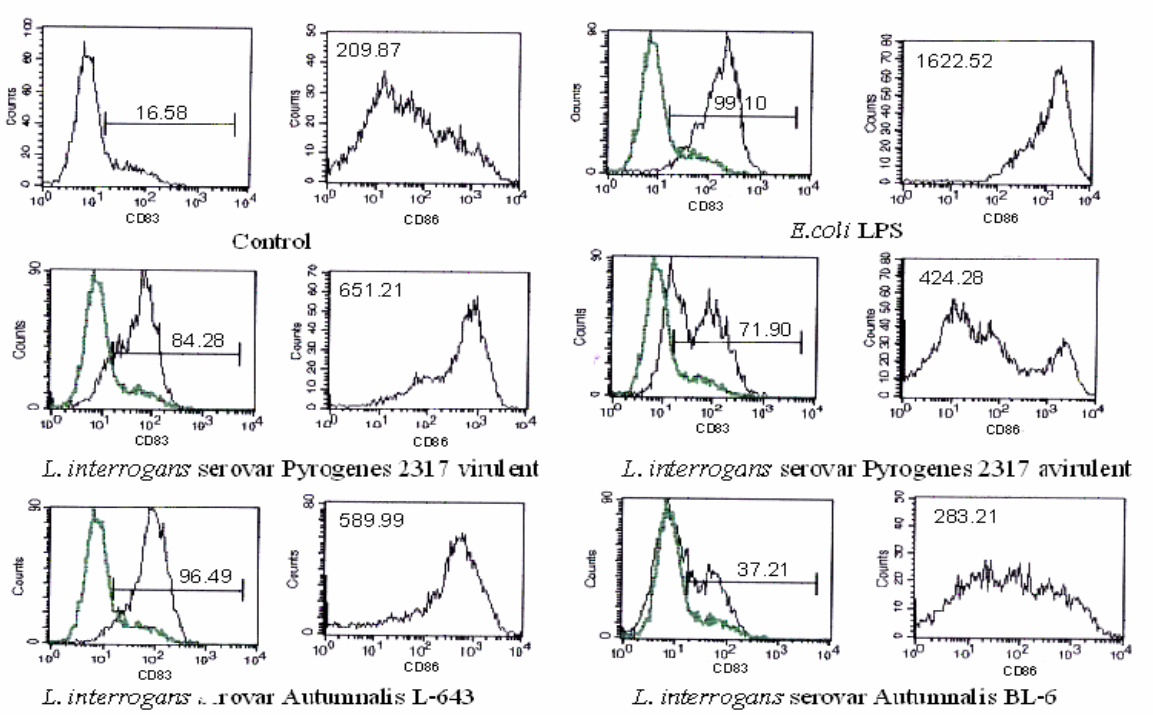


Figure 22 Histogram of CD83 and CD86 expression on immature DCs. DCs maturation were detected with two colors staining after 2 days stimulation (A-E). CD83 expression were presented as percent of compares between immature DC (gray) and mature DCs (black). CD86 expression (black) were presented as mean fluorescent intensity (MFI).

5. Cytokine productions by *L. interrogans* activated MoDCs

Supernatants of 2 days leptospiral-MoDCs co-culture were determined for cytokines secretion. Individual donor showed similar trend of response to leptospiral activation that higher cytokines secretion for virulent Pyrogenes 2317 and less extend for avirulent strain, serovar Autumnalis BL-6 and L-643, respectively.

For IL-12p70 cytokine secretion, MoDCs from donor 1 and 2 were induced to secrete higher IL-12p70 when activated with virulent *L. interrogans* serovar Pyrogenes 2317 though they were induced low IL-12p70 secretion when activated with *E.coli* LPS control (Table 10 A, Figure 23 A). MoDCs from donor 1 and 2 secreted IL-12p70 when were activated with serovars Pyrogenes 2317 avirulent strain and serovar Autumnalis BL-6 strain also. MoDCs from Donor 4 and 5 were induced to secrete low level of IL-12p70 when stimulated with virulent Pyrogenes 2317 strain but not with the others leptospiral species. However, MoDCs from donor 3 could be induced to secret very low or undetectable IL-12p70 secretion when activated with either LPS control or leptospires. In deed, virulent *L. interrogans* serovar Pyrogenes 2317 strain stimulated MoDCs from any donors to secrete higher level of IL-12p70 than when activated with avirulent strain. In contrast, *L. interrogans* serovar Autumnalis L-643 could not induce IL-12p70 secretion in all MoDCs from donors.

For TNF-α secretion, virulent *L. interrogans* serovar Pyrogenes 2317 induced MoDCs from all donors to secrete the highest TNF-α. A much lower extend of the cytokines secretion was obtained when MoDCs from all donors were induced by avirulent strain, serovar Autumnalis BL-6 and especially serovar Autumnalis L-643 which induced very low or undetectable TNF-α secretion (Table 10 B, Figure 23 B).

Then, IL-10 secretion was further investigated since serovar Autumnalis L-643 induced MoDCs from all donors to secrete low or undetectable levels of either IL-12p70 or TNF-α. All isolated *L. interrogans* induced low level of IL-10 production especially serovar Autumnalis L-643 in all donors even though donor 4 and 5 induced high IL-10 secretion when activated with *E.coli* LPS (Table 10 C, Figure 23 C).

In conclusion, all *L. interrogans* serovars could induce DC maturation but different in cytokine production in individual donor. Virulent *L. interrogans* serovar Pyrogenes 2317 induced concomitant IL-12p70 and TNF-α as well as low level of IL-10 production by MoDCs whereas avirulent strain and serovars Autumnalis BL-6

induced MoDCs from some donors to produce these cytokines in lower level. In contrast, *L. interrogans* serovar Autumnalis L-643 strain induced lower or undetectable levels of these cytokines.

Table 10 Cytokine secretion by *L. interrogans* activated MoDCs

A. Concentration of IL-12p70 in pg/ml

Sample	Donor				
	1	2	3	4	5
medium	45.93	0.00	0.00	0.00	0.00
LPS	146.47	452.73	60.89	541.60	955.68
Pyrogenes (vi)	479.56	1620.39	62.72	280.76	242.35
Pyrogenes (avi)	411.90	960.21	7.90	9.16	0.00
Autumnalis L-643	0.00	6.60	0.00	0.00	0.00
Autumnalis BL-6	308.08	725.82	0.00	0.00	16.25

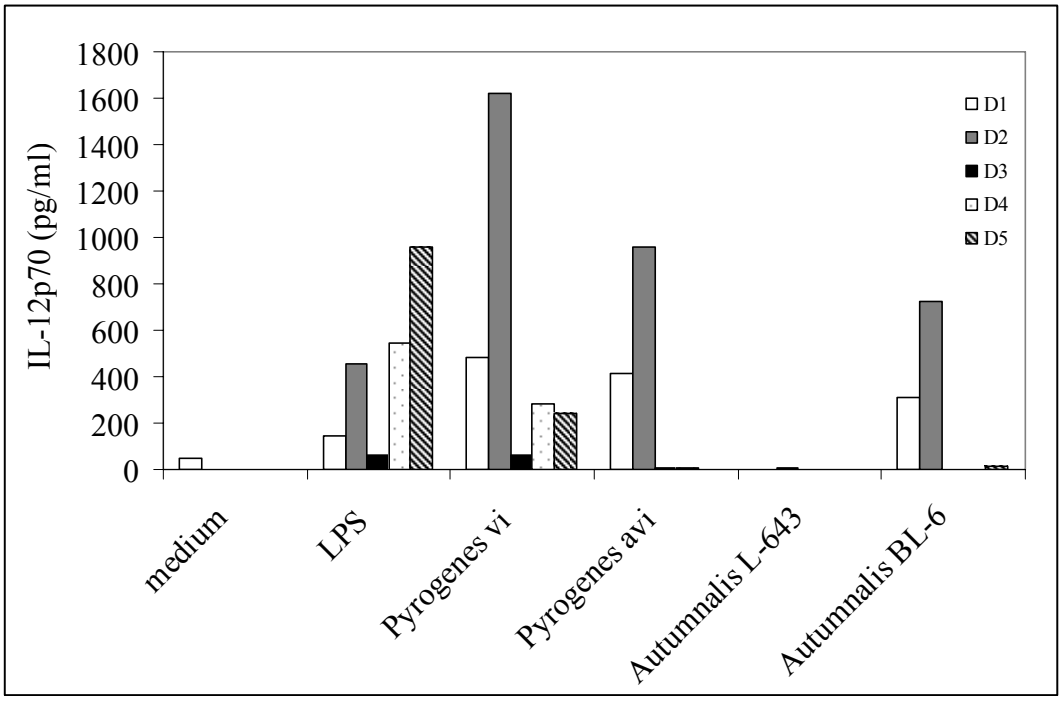
B. Concentration of TNF-α in pg/ml

Sample	Donor				
	1	2	3	4	5
medium	0.00	2497.36	0.00	0.00	0.00
LPS	2211.14	2835.98	9232.74	13758.94	8194.64
Pyrogenes vi	12951.86	15087.46	18144.30	4785.44	1421.38
Pyrogenes avi	8136.58	5352.84	3137.84	2539.00	445.56
Autumnalis L-643	660.38	610.10	2552.86	0.00	0.00
Autumnalis BL-6	8398.50	4771.82	701.80	1399.18	871.64

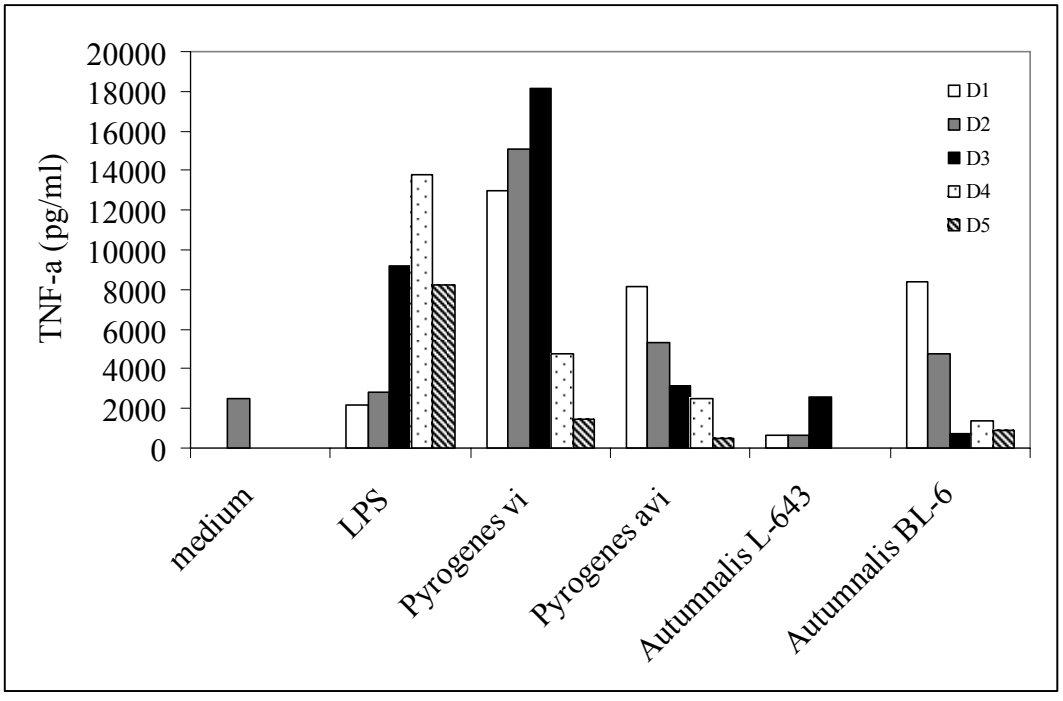
C. Concentration of IL-10 in pg/ml

Sample	Donor				
	1	2	3	4	5
medium	3.68	34.695	28.33	30.715	37.085
LPS	87.365	79.365	499.67	1847.125	2090.7
Pyrogenes vi	498	443.97	62.595	228.32	371.29
Pyrogenes avi	523.87	311.4	797.515	158	107.38
Autumnalis L-643	11.625	40.27	52.225	18.785	21.965
Autumnalis BL-6	485.505	54.615	49.83	179.77	197.545

A


IL-12p70 (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
D1
D2
D3
D4
D5
medium
LPS
Pyrogenes vi
Pyrogenes avi
Autumnalis L-643
Autumnalis BL-6

B


TNF-a (pg/ml)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
D1
D2
D3
D4
D5
medium
LPS
Pyrogenes vi
Pyrogenes avi
Autumnalis L-643
Autumnalis BL-6

C

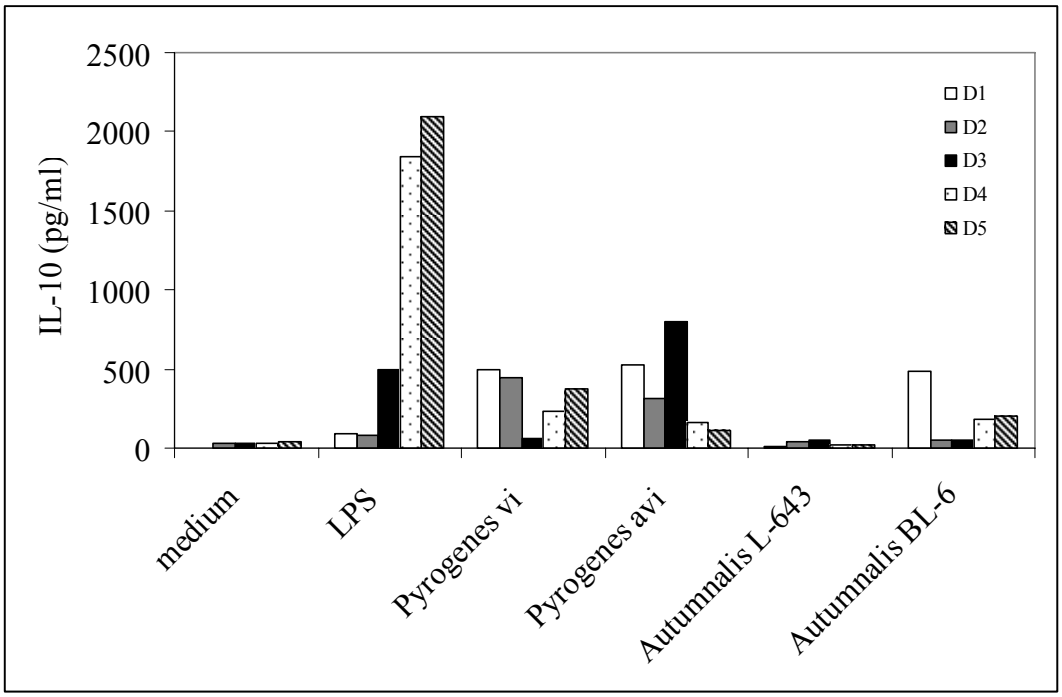
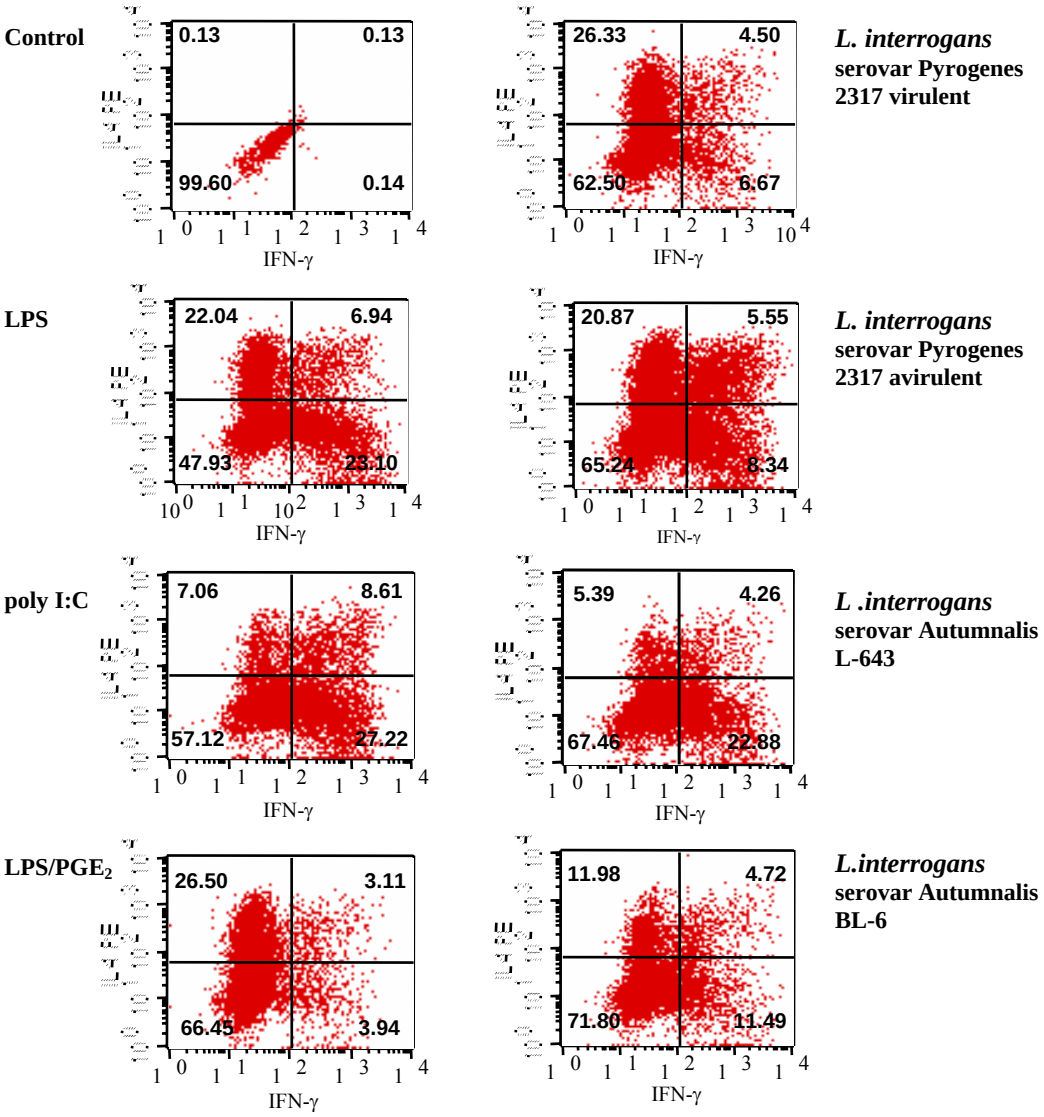


Figure 23 Cytokine production by *L. interrogans* activated MoDCs. Cell free culture supernatants were collected and pooled from triplicate wells and then measured for IL-12p70 (A), TNF-α (B) and IL-10 (C) by ELISA.

6. DCs cocultured with *L. interrogans* induced mixed Th1/Th2 responses

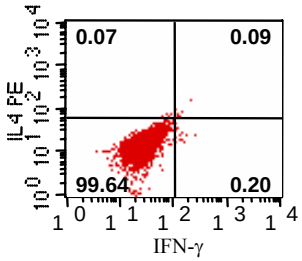
To determine the function of MoDCs in T cells activation, two days *L. interrogans* stimulated MoDCs were added to naïve T cells at ratio 1:4. Known stimuli for Th1 (polyI:C), Th2 (LPS/PGE_2) and mixed Th1/Th2 (*E. coli* LPS) were included in all experiments. The cells were further cultured for 12 to 14 days. Then, Th1 and Th2 cell profiles were assessed by restimulated T cells with PMA and ionomycin and stained for intracellular IL-4 and IFN-γ cytokines. The results showed that these MoDCs could enhance naïve T cells proliferation and cytokine production. However, the priming of leptospires did not impose specific T cells polarization compared to controls of individual 7 donors (Figure 24 A-G). Activated T cells developed mixed population of IL-4 and IFN-γ producing T cells depend on both DCs and naïve T cells donors. Most of donors showed different in T cells responses. *L. interrogans* serovar Pyrogenes 2317 virulent strain tended to produced Th1 in 1 donor, Th2 for 1 donor and mix Th1/Th2 in 5 donors whereas avirulent strain tended to produced Th1 in 3 donors, Th2 in 1 donor and mix Th1/Th2 in 3 donors. *L. interrogans* serovar Autumnalis L-643 strain tended to produced Th1 in 3 donors, Th2 for 1 donor and mix Th1/Th2 in 3 donors whereas BL-6 strains tended to induce Th1 in 4 donors, Th2 in 1 donor and mixed Th1/Th2 in 2 donors. Thus, *L. interrogans* induced naïve T cells to produced either Th1, Th2 or mixed Th1/Th2 depend on donors.

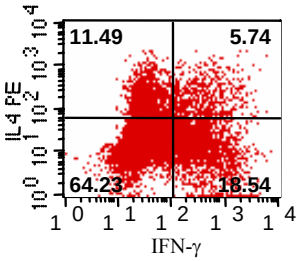
A


Control
0.13
0.13
99.60
0.14
IFN-γ
26.33
4.50
62.50
6.67
IFN-γ
L. interrogans
serovar Pyrogenes
2317 virulent
LPS
22.04
6.94
47.93
23.10
IFN-γ
20.87
5.55
65.24
8.34
IFN-γ
L. interrogans
serovar Pyrogenes
2317 avirulent
poly I:C
7.06
8.61
57.12
27.22
IFN-γ
5.39
4.26
67.46
22.88
IFN-γ
L .interrogans
serovar Autumnalis
L-643
LPS/PGE2
26.50
3.11
66.45
3.94
IFN-γ
11.98
4.72
71.80
11.49
IFN-γ
L.interrogans
serovar Autumnalis
BL-6

B

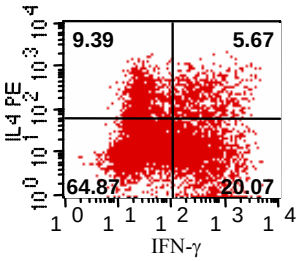
Control

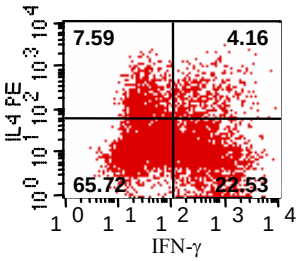




L. interrogans **serovar Pyrogenes 2317 virulent**

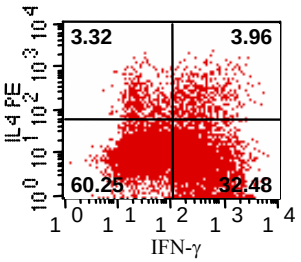
LPS

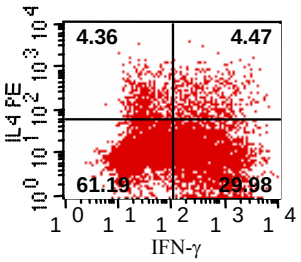




L. interrogans **serovar Pyrogenes 2317 avirulent**

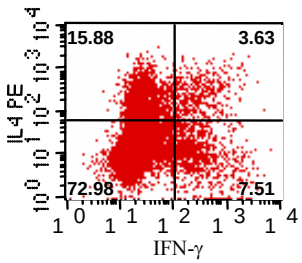
poly I:C

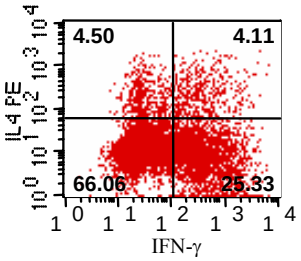




L. interrogans **serovar Autumnalis L-643**

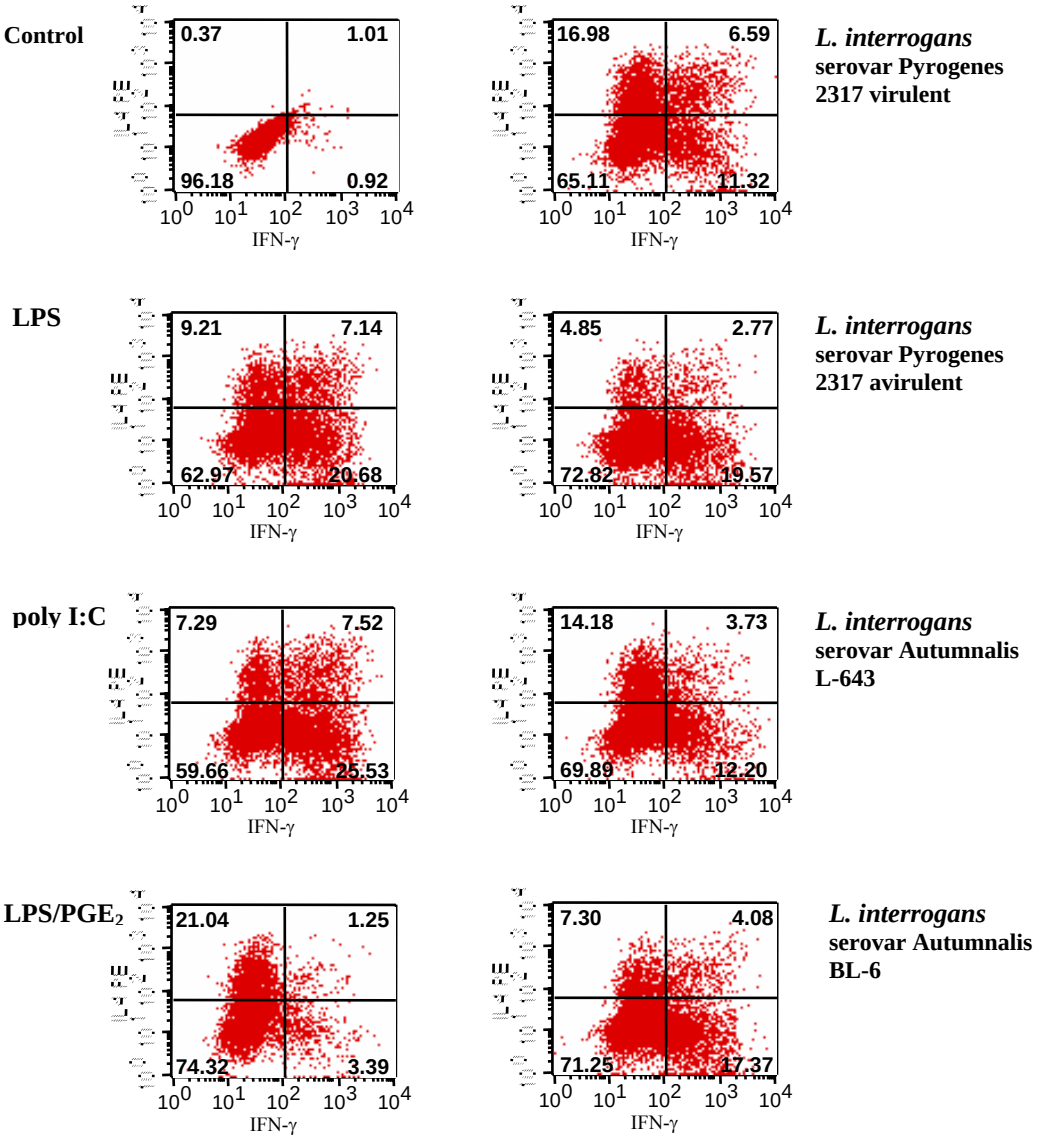
LPS/PGE$_2$





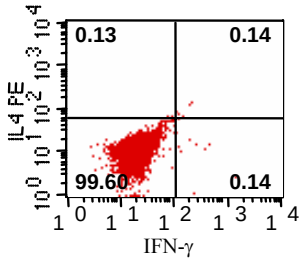
L. interrogans **serovar Autumnalis BL-6**

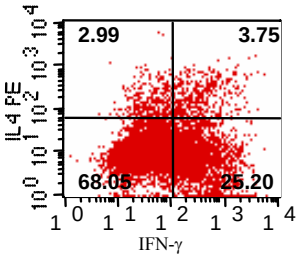
C


Control
0.37
1.01
96.18
0.92
16.98
6.59
65.11
11.32
L. interrogans serovar Pyrogenes 2317 virulent
LPS
9.21
7.14
62.97
20.68
4.85
2.77
72.82
19.57
L. interrogans serovar Pyrogenes 2317 avirulent
poly I:C
7.29
7.52
59.66
25.53
14.18
3.73
69.89
12.20
L. interrogans serovar Autumnalis L-643
LPS/PGE2
21.04
1.25
74.32
3.39
7.30
4.08
71.25
17.37
L. interrogans serovar Autumnalis BL-6
IFN-γ

D

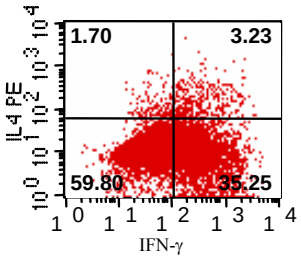
Control

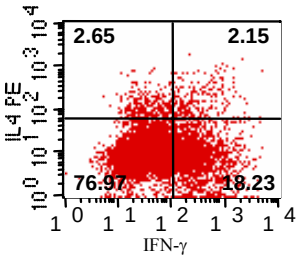




L. interrogans **serovar Pyrogenes 2317 virulent**

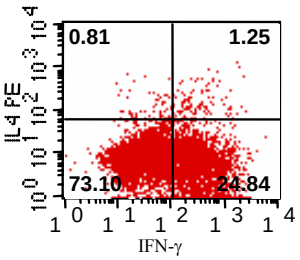
LPS

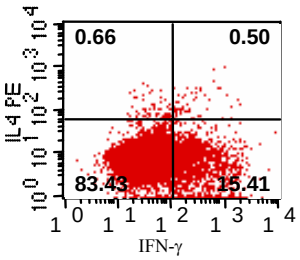




L. interrogans **serovar Pyrogenes 2317 avirulent**

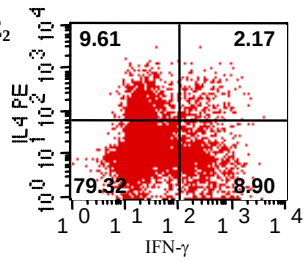
poly I:C

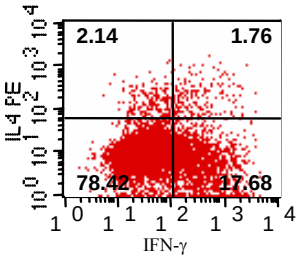




L. interrogans **serovar Autumnalis L-643**

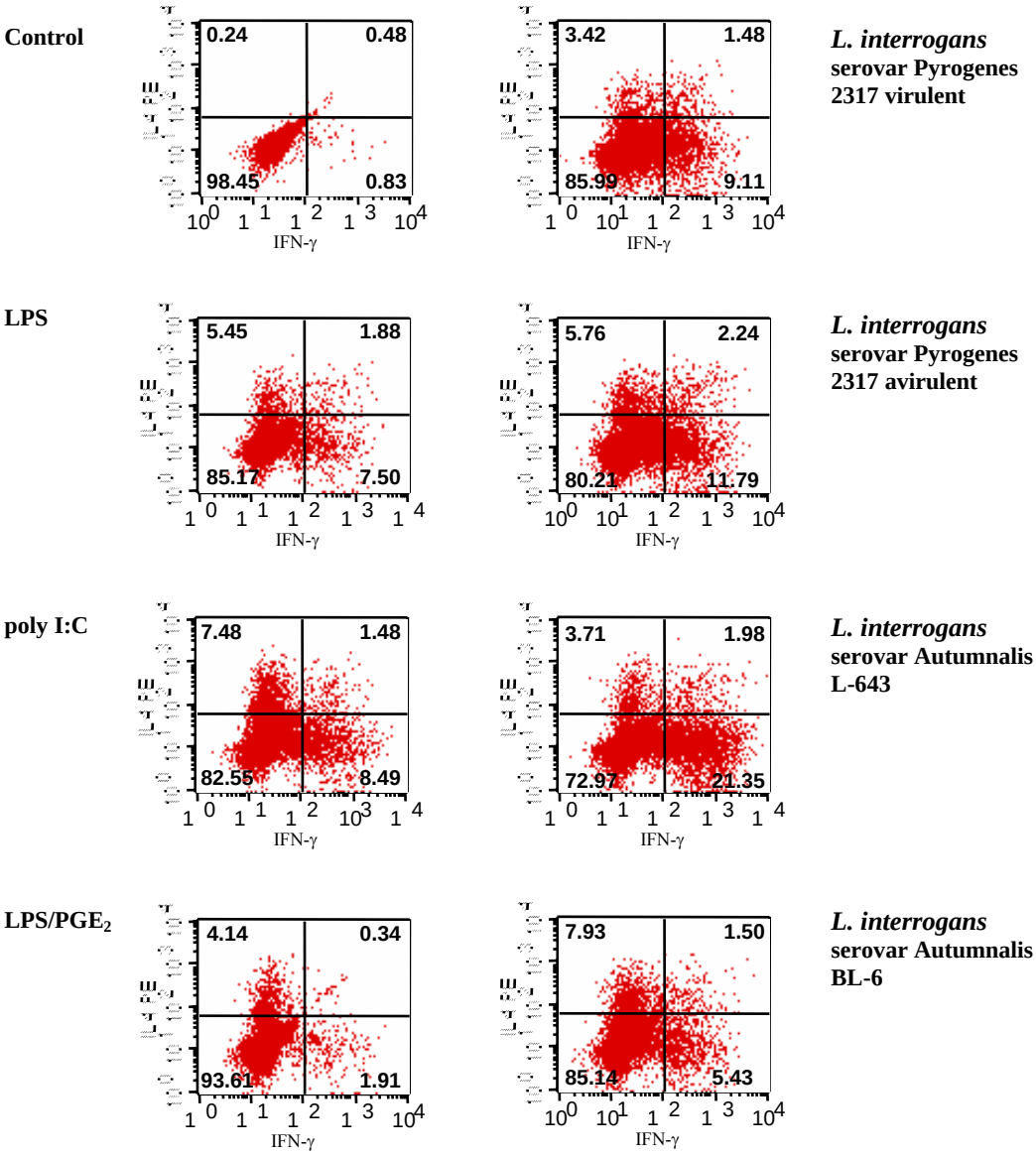
LPS/PGE$_2$





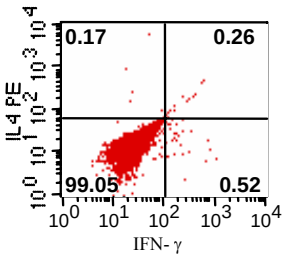
L. interrogans **serovar Autumnalis BL-6**

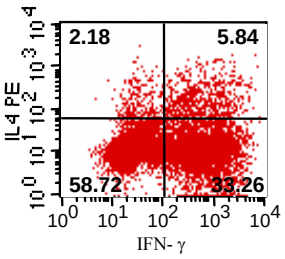
E


Control
0.24
0.48
98.45
0.83
3.42
1.48
85.99
9.11
IFN-γ
L. interrogans serovar Pyrogenes 2317 virulent
LPS
5.45
1.88
85.17
7.50
5.76
2.24
80.21
11.79
IFN-γ
L. interrogans serovar Pyrogenes 2317 avirulent
poly I:C
7.48
1.48
82.55
8.49
3.71
1.98
72.97
21.35
IFN-γ
L. interrogans serovar Autumnalis L-643
LPS/PGE2
4.14
0.34
93.61
1.91
7.93
1.50
85.14
5.43
IFN-γ
L. interrogans serovar Autumnalis BL-6

F

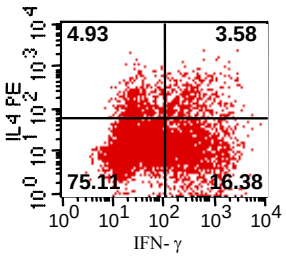
Control

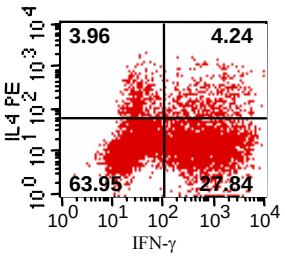




L. interrogans **serovar Pyrogenes 2317 virulent**

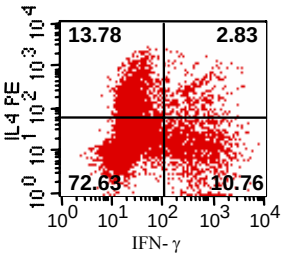
LPS

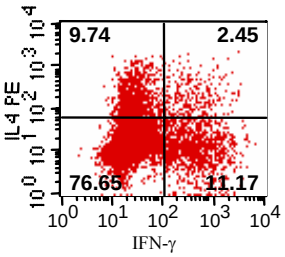




L. interrogans **serovar Pyrogenes 2317 avirulent**

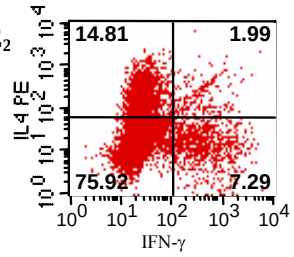
poly I:C

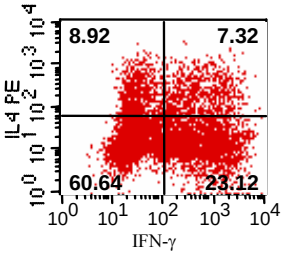




L. interrogans **serovar Autumnalis L-643**

LPS/PGE$_2$





L. interrogans **serovar Autumnalis BL-6**

G

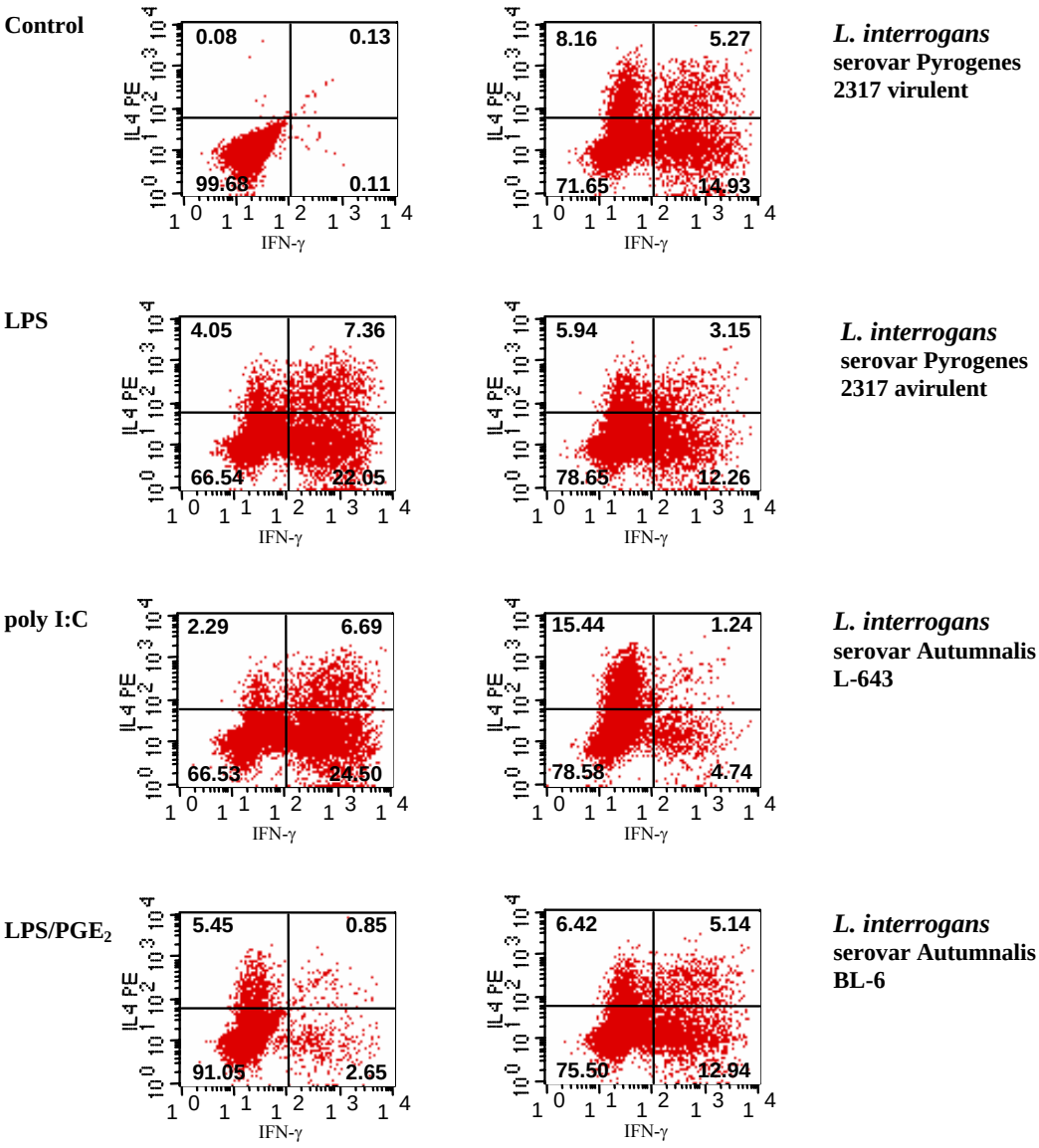


Figure 24 Effect of naive T cells activation by *L. interrogans*. DCs were primed with *L. interrogans* serovar Pyrogenes 2317 virulent and avirulent strains and *L. interrogans* serovar Autumnalis L-643 and BL-6 strains, and stimulated naïve T cells. *E. coli* LPS (5 μg/ml), polyI:C (100 μg/ml) or LPS/PGE_2 (100 μg/ml) were used as positive controls. T cells were restimulated with PMA (10 ng/ml) and iomomycin (1 μg/ml) and stained for intracellular IL-4 and IFN-γ cytokines.

CHAPTER VI
DISCUSSION

Leptospira is a global zoonosis caused by *L. interrogans*. In recent years, leptospirosis has dramatically increased in Thailand especially in the north and northeastern regions [31]. Leptospiral infection serovars were varied in different areas, mostly depending on the predominant infected serovars of reservoirs. The clinical signs are vary from mild to severe disease and can be fatal if not properly managed.

Leptospiral pathogenesis is not well understood but believed to be the result of both immunopathological of the host immune response and the leptospiral itself [11]. The molecular mechanisms that control the immune response to *L. interrogans* are not completely understood. *Leptospira* has been studied in several aspects such as expression of antigens, cell interaction and immune responses both *in vitro* and *in vivo*. There are differently response between nonpathogenic and pathogenic strains. Virulent leptospires which are clinical isolates can survive intracellulary by escape phagocytosis and induce apoptosis while avirulent leptospires are rapidly cleared [2, 143]. One factor may be related to difference in surface expression of *Leptospira*. For example, fibronectin binding protein is expressed only in virulent leptospires and not found in avirulent and nonpathogenic strains [80]. Fibronectin may act as a bridge between domain of integrin on cells and leptospires. CR3 receptor has been reported as the receptor for both pathogenic and nonpathogenic leptospires and acts as adhesion molecule rather than endocytose receptor [85].

There are several reports concerning interaction between *Leptospira* and immune cells such as monocytes/macrophages that phagocytose and eliminate leptospira. Moreover, peripheral blood mononuclear cell (PBMC) activated with heat-killed whole cell *L. interrogans* induced Th1 response by secretion of TNF-α, IFN-γ and IL-12 which may play an important role in the leptospiral protective [100]. However, there is no report about the response of DCs against *Leptospira.*

DC-SIGN is well-known type II C-type lectin (CLR) that play a crucial role in pathogen recognition and immune evasion on DCs. DC-SIGN is abundantly expressed on DCs present on skin leading to the proposal that DCs residing at the primary site of pathogenic infection. Clustered distribution or microdomain on plasma membrane (lipid raft) is essential to enhance the interaction as well as internalization efficiently of DC-SIGN pathogen complexes [127].

Several pathogens are captured by DC-SIGN to gain entry into DCs, to be transported to T cells or to induce immune escape such as Ebola virus, Cytomegalovirus, *Leishmania*, *M. tuberculosis* , HHV8 and HIV-1 [144-149]. Upon DC-SIGN binding, DC-SIGN-ligand complexes were internalized to a large late endosomal compartment, dissociate antigen then recycling to cell surface [114].

DC-SIGN is specific to high mannose N-linked oligosaccharides and mediated the strongest binding to ManGlcNAc and FucGlcNAc [128]. DC-SIGN also binds to other sugars that lack mannose-related epitopes, such as Le^x structure (Galβ1-4(Fucα1-3)GlcNAc) [128]. Recently, Zhang *et al* [150] reported that GlcNAc sugar within the core LPS is a major target of DC-SIGN binding.

Lectins are often complex, multidomain proteins, which sugar binding activity can usually be ascribed to a single CRD. Lectins natural ligands are presumably glycans of high N-linked glycans that differ in specificity. For instance, ConA, a typical lectins, is specific for both mannose and glucose whereas other lectins bind galactose or glucosamine or their derivatives [138]. *Leptospira* cell surface contains high carbohydrate components [11]. Biotinylated lectins recognized *Leptospira* in different specific. Overall, *Leptospira* contains high mannose N-linked glycan as major carbohydrate on their structure as shown by ConA recognition and some of other carbohydrate molecules such as Galβ1-4GlcNAcβ1-R and GlcNAcβ1-4GlcNAcβ1-4GlcNAc that recognized by RCA_{120} and WGA. This data might relate to mannobiose unit which an antigenic epitope of nonpathogenic *L. biflexa* Patoc I and genus specific antigen as previously reported [140-142,151]. From these characteristics, *Leptospira* which contains high mannose as a major carbohydrate might also be recognized by DC-SIGN receptor.

Soluble DC-SIGN-Fc IgG which is used to screening DC-SIGN binding properties suggested that *L. interrogans* bound specifically to soluble DC-SIGN-Fc IgG either whole cells or secreted antigens of *L. interrogans*. Since leptospiral secreted antigen contains carbohydrates component also. Moreover, a representative live and paraformaldehyde-killed *L. interrogans* serovar Pyrogenes 2317 virulent strain could equally bound to soluble DC-SIGN-Fc IgG. This results suggest that either live or killed leptospira can bind DC-SIGN. The viability has no effect on *L. interrogans* binding to DC-SIGN and the further experiments were performed by using killed bacteria.

Compare binding on DC-SIGN expressing cell showed that *L. interrogans* serovars bound DC-SIGN. *L. interrogans* showed higher binding on K-SIGN which are DC-SIGN transfected K-562 myelogenous leukemia cells and constitutively expressed DC-SIGN rather than K-562 cells control. *L. interrogans* bound DC-SIGN stronger on cellular DC-SIGN than soluble DC-SIGN-Fc IgG, especially *L. interrogans* serovar Autumnalis BL-6 strain. These might be due to the difference in composition of carbohydrate components on leptospiral surfaces and also structures of DC-SIGN. In general, DC-SIGN binding achieved through tetameric form on cell surface which facilitate higher degree of binding [127]. Soluble DC-SIGN-Fc IgG has a binding site on each arm of IgG chimera and contains two binding sites. Tetameric form of DC-SIGN on cell surface may increase the affinity for binding with ligands on bacteria better than monomeric or dimeric forms on soluble DC-SIGN-Fc IgG.

In deed, avirulent of serovar Pyrogenes 2317 showed more efficient on DC-SIGN binding and also showed more affectionate on DC-SIGN blocking than virulent strain and serovar Autumnalis. Whereas serovar Autumnalis has less effect than serovar Pyrogenes on DC-SIGN binding in the present of inhibitors. This might be involved in the other receptor rather than DC-SIGN on the cells since serovar Autumnalis showed high binding to K-562 cells as well.

For MoDCs, *L. interrogans* bound to MoDCs higher than K-SIGN. This result might consistent with the fact that DCs express more receptors than K-SIGN which derived from K-SIGN that does not express some receptor found on MoDCs such as mannose receptor [115, 152].

Interstingly, the blocking agents, especially EDTA, have more affected on K-SIGN binding of serovar Pyrogenes more than serovar Autumnalis that differ from MoDCs which serovar Autumnalis affected from these blocking agent more than serovar Pyrogenes. *L. interrogans* Autumnalis interaction to MoDCs depends on DC-SIGN more than serovar Pyrogenes. These phenomena might be the cooperation of the other undefinded receptors that expressed on MoDCs facilitate difference binding. For example, CR3 receptor on MoDCs and fibronectin-binding protein on *L. interrogans* might enhance the adherence of pathogenic bacteria to the cells. Furthermore, mannan, EDTA, Ca^{2+} chelator, and anti-DC-SIGN antibody, AZN-D1, also inhibit the function of other receptors on MoDCs. Thus, *L. interrogans* binding might involved in mannose receptor and C-type lectin or other undefinded receptor that need Ca^{2+} molecule on the binding site.

Moreover, MoDCs showed more effective recognition to avirulent *L. interrogans* serovar Pyrogenes 2317 than virulent strain. It might be possible that avirulent leptospiral could be clearly better than virulent leptospiral. However, the binding and blocking efficiency of *L. interrogans* were varied depend on MoDCs from individual donors that might be different in genetic background. Thus, on DC-SIGN expressing cells either K-SIGN or MoDCs demonstrated that *L. interrogans* binding used DC-SIGN as one of a receptor and, at least, C-type lectin involved in the recognition of *Leptospira*.

Changing in surface component of virulent *L. interrogans* after *in vitro* cultivation has been reported [2, 153]. Culturing can cause changing in undefined molecules of LPS and outer membrane proteins expression. *In vivo* and *in vitro* models showed that the infectivity of virulent leptospires did not correlate to loss of virulence since low passaged leptospiral that progressive loss virulence or nonpathogenic strains could infected target cells when extended time of infection [2]. Natarajaseenivasan *et al.* [154] reported the correlation between serovar-specific and complication of leptospirosis. However, this report was contrast to Faine *et al.* [11] which showed that there was no correlation of serovars and clinical complication. Several strains that harboring similar surface antigens are vary in severity ranging from mild to severe illness. Moreover, these might associated with some differences in recognition and activation to host cells. For example, Pyrogenes has 2 contact genes involved in

integral membrane proteins and linked to galactosyltransferase gene but Autumnalis has only one gene and lack the galactosyltransferase gene [23]. However, on the basis of sequence identity, it would be reasonable that subunit structures of Pyrogenes and Autumnalis are closely related. Then, the serovar specific and clinical complication still controversial, both the virulence properties of *L. interrogans* strain and host factors may strongly influence the clinical outcome of the infection.

DC maturation trigger by TLRs signaling leads to up-regulation of MHC-molecules, costimulatory molecules and cytokine productions whereas CLRs operate antigen capture and uptake. DC-SIGN has been reported in *H. pyroli* studies that it was not involved in DCs maturation and after blocking DC-SIGN binding, DCs maturation did not alter [135].

Spirochetes, *Treponema palliduma* and *Borrelia burgdoferi* which causative of syphilis and Lyme diseases, can also activate DC maturation *both in vitro* and *in vivo* [155-158]. They are phagocytosed by DCs as early as 2 h after activation, activate DC maturation and enhance T cell proliferation. Moreover, they are potent activators to up-regulate DC-SIGN on DCs and monocytes/macrophages and induce IL-6, IL-10, IL-12, IFN-γ productions [155]. The activation was involved in their or their products of lipopeptides that displayed significant increase in surface expression of TLR1, TLR2, and TLR4 and also cytokine productions [155].

Cross talk between DC-SIGN and TLR signaling pathways during microbial sensing and recognition has been documented [115, 130]. Co-expression of DC-SIGN served as an attachment receptor that facilitates TLR2 signaling therefore resulting in increasing of NF-κB activation by spirochetes lipoprotein activation [155]. Recent reports showed that *L. interrogans* could activate immune response by TLR-2 and TLR-4 [88, 89, 91, 92]. TLR-2 acted as receptor for outer membrane proteins such as LipL32 of pathogenic leptospires whereas TLR-4 could activate immune response with other undefined molecules. These data confirmed that both leptospiral LPS and lipoprotein expose on the outer membrane. These lipoproteins did not block LPS binding to macrophages, suggested that cell activation may connect through a different pathway. In these manners, it is likely that TLRs might involved in DCs maturation after recognize leptospiral components either LPS or lipoprotein.

Our results demonstrated for the first time that *L. interrogans* serovars Pyrogenes and Autumnalis activated DC maturation by expressed CD83 maturation marker molecule and CD86 costimulatory molecule in comparable levels. However, *L. interrogans* serovar Autumnalis L-643 strain activated partial MoDCs maturation as low CD83 maturation marker in MoDCs from one donor. In contrast, *L. interrogans* activated MoDCs to produce differences in cytokine release. High amount of IL-12 and TNF-α were produced only after stimulation with *L. interrogans* serovar Pyrogenes 2317 virulent strain though bound MoDCs less than avirulent strain. A possible explanation for this observation may be related to the lost of integrity of the bacterial virulence on membrane during *in vitro* culture. The virulent strain may have variant moieties with pronounced immunostimulating activity better than avirulent strain. *L. interrogans* serovar Autumnalis L-643 and BL-6 strains bound and activated co-stimulatory molecules on MoDCs in comparable levels with Pyrogenes 2317 virulent strain but they initiate very low or undetectable IL-12p70 and TNF-α. Interestingly,*L. interrogans* serovar Autumnalis L-643 which was isolated from blood sample of deceased leptospirosis patient did not showed TNF-α production by MoDCs in this study. This result was unexpected since high level of proinflammatory cytokine TNF-α production in response to *L. interrogans* has profound consequences for the severity and mortality of leptospirosis [41, 159]. The reason for this non production of TNF-α is unknown. Cytokine IL-10 which involved in immune suppression was detected at low levels when activated MoDCs with *L. interrogans* serovar Pyrogenes 2317 both virulent and avirulent and serovar Autumnalis BL-6 strains . However, *L. interrogans* serovar Autumnalis L-643 was not induced IL-10 secretion in MoDCs from all donor as well. Then, IL-10 was not involved in IL-12 production inhibition. Thus, the factor that related to severity of patient who infected with *L. interrogans* serovar Autumnalis L-643 was unknown and might involved host immune status. This finding indicates that, although the phenotypic outcome of the interaction of *L. interrogans* serovars with DC maturation are similar, the functional implications in term of cytokines production are distinguish.

There were reports demonstrating that human PBMC activated with heat-killed *L. interrogans* serovars Copenhageni and Rachmani and *L. borgpetersenii* could induced the production of Th1 cytokine which were IL-12p40, TNF-α and IFN-γ [96,

98, 99]. The production of IFN-γ is largely dependent on IL-12 which are mainly produced by CD4+ T cells both in *in vitro* and *in vivo* [97-100]. These results were similar to *in vivo* cytokine mRNA study [160].

The relationship between IL-12 and TNF-α remains unclearly defined. Chierakul *et al.* [161] reported that there were high plasma concentration of IL-12p40, and TNF-α in adults Thai leptospirosis patients. Indeed, IFN-γ was detected at low level and IL-12p70 was detected in a small number of leptospirosis patients. This likely due to the fact that bioactive IL-12p70 is made lower than IL-12p40.

Naive CD4+ T cell is able to develop into either a Th1 or Th2 cell that secretes predominantly IFN-γ or IL-4, respectively. DC derived IL-12 can potently stimulates IFN-γ production by naïve T helper cell, especially by intracellular pathogens. Although *L. interrogans* bound to both soluble and cellular DC-SIGN, they were different in initiation T cell responses. The priming of leptospiral did not imposed specific T cells polarization but mixed Th1/Th2 response as judged by the production of IFN-γ and IL-4 by naïve T cells of individual donors. The reason for this phenomenon is unclear but might include host genetic background variability either DCs or naïve T cells since LPS as mixed Th1/Th2 activator also biased to Th1 response for some donors.

Even though both virulent and avirulent strains of *L. interrogans* serovar Pyrogenes 2317 and *L. interrogans* serovar Autumnalis BL-6 strain induced MoDCs to produce high level of IL-12p70 production in some donors while *L. interrogans* serovar Autumnalis L-643 undetectable IL-12p70 production at all donors, they all stimulated mixed Th1/Th2 intracellular cytokine production and tend to bias to Th1 response. *L. interrogans* serovar Autumnalis L-643 might be mediated by IL-12 independent residual pathway that related to CD86 molecule activation to induce T cell response as reported for Mycobacterium [162, 163]. Since MoDCs from individual donors activated by this strain induced highly expression of CD86 costimulartory molecule even though low expression of CD83 maturation marker.

When compared intracellular cytokine production using MoDCs from the same donors but different naïve T cells donors or vi versa, difference T cell responses were induced. These might be adaptation of leptospiral to balance immune response or involvement of host variability. The mechanism is not clearly understood.

Targeting DC-SIGN can modulate the immune response in either Th1 or Th2 direction. The mechanism is how DC-SIGN directs T cell proliferation and response are currently unknown. The possible mechanism may due to immunological synapse formation between DC- T cells [164]. DC-SIGN may exert initial transient contact with ICAM-3 on T cells and prolong cell-cell contact and thereby prolong TCR signaling that important signal for T cells proliferation.

It has been suggested that the polarization may depend on the balance between CLRs and TLRs activation [134]. Several pathogens such as *Neisseria meningitis*, *Mycobacterium*, *Leishmania* and *Helicobacter* manipulate balance of Th1/Th2 to cause chronic infections [115, 135, 146, 165]. Liana *et al*. [165] reported that LPS-deficient *N. meningitis* which was very poorly internalized, activated DC maturation and it targeted DC-SIGN that skew Th1 response. Some bacteria occupy DC-SIGN and modulate suppression of TLRs signaling such as *M. tuberculosis* binds DC-SIGN and suppresses TLR-4 then interfere TLR signaling [115]. *Leishmania mexicanan* also binds DC-SIGN and favors Th2 response rather than Th1 and causes chronic infection [146]. *H. pylori* which contains less LPS toxin activity than other LPS from other gram-negative bacteria alter the Th1/Th2 environment of the host to evade immune responses and suppressed IL-12 p70 production also tends to induce Th2 response [135, 166]. Strikingly, the same results have been shown for LPS from *P. gingivalis* that contains unusually branched and long fatty acids that functionally interacts with both TLR2 and 4 and favors Th2 response [167, 168]. The characteristic of *H. pylori* and *P. gingivalis* are similar to *Leptospira* that has unusual fatty acid and lower biological effect of LPS than gram negative bacteria [11]. *Leptospira* also cause chronic infection as occur Uveitis in human and horse [28, 83]. The severity of Weil's disease has been reported that may be associated with the intensive of humoral immunity to leptospires since though leptospirosis patients showed high IgG but they occurred worse pulmonary symptoms [169]. In contrast, induction of Th1 response by secretion of TNF-α, IFN-γ and IL-12 may play an important role in the leptospiral protective [96, 97, 170]. Then, it is possible for pathogenic leptospires to balance Th1/Th2 cytokine production for their survival in host.

DC-SIGN can bind various ligands in different ways, which have consequences for how the pathogen is processed by DCs and activate T cells response to either immune activation or immunopathogenesis. However, this study did not extend direct role *L. interrogans* and DC-SIGN on the role of naïve T cells polarization.

In general, this study identified DC-SIGN as cell receptor for leptospiral recognition on human DCs. This interaction between *L. interrogans* and DCs induced DCs maturation and provided T cell proliferation and cytokines productions in mixed Th1/Th2 responses. The further study on receptors and molecular mechanism in leptospira pathogenesis will provide more information and guide for new concept of therapeutic and vaccine targets.

CHAPTER III
CONCLUSION

Several reports show that specialized C-type lectin receptor called DC-Specific ICAM-3-Grabbing Non-integrin (DC-SIGN) or CD209 are expressed on DCs is the crucial step of DCs. DC-SIGN binding is likely to have important effects on the immunological and pathological events associated with microorganisms that induce either response or evasion of the immune system. The present study was performed to 1) evaluate the interaction of *L. interrogans* and DCs 2) investigate the receptor involved in bacteria binding on DCs. *L. interrogans* and DCs interaction was studied in term of cell binding, DC maturation, cytokine production induced by DCs and T cell response whether it is Th1 or Th2 polarization.

The present study provide the first information of *L. interrogans* interaction on DCs and the induction of immune response *in vitro*. First, Leptospira contain mannose as a common backbone on cell surface. Second, surface DC-SIGN expression diffusely over the cell surface of a DC is one of *Leptospira* recognition receptor. Third, the recognition to DC-SIGN is specific since competing ligands such as anti-DC-SIGN antibody and mannan abrogated binding. EDTA also interferes the binding of ligand since DC-SIGN depends on the binding of two Ca^{2+} ions to carbohydrate recognition domains (CRD). Forth, despite *L. interrogans* do stimulate DCs maturation as judged by up-regulation of costimulatory molecules, CD83 and CD86, they are strikingly different on IL-10, IL-12p70 and TNF-α production. *L. interrogans* serovar Pyrogenes both virulent and avirulent strains stimulated DCs to produce higher IL-10, IL-12p70 and TNF-α higher than serovar Autumnalis BL-6 strain but serovar Autumnalis L-643 strain did not stimulate DCs to produce both cytokines. Only high amounts of IL-12p70 in the supernatants of MoDCs stimulated with *L. interrogans* serovar Pyrogenes 2317 both virulent and avirulent strains were confirmed by the IFN-γ production by T cells though virulent strain showed higher levels. Surprisingly, TNF-α that involved in severity of leptospirosis did not detected

in *L. interrogans* serovar Autumnalis L-643 which was isolated from blood sample of patient who deceased from leptospirosis. The reason of this is unknown. Finally, *L. interrogans* activate naïve T cell response in non-specific polarization form as demonstrate by mixed Th1/Th2 responses and tend to bias Th1 response and IL-12 independent. The mixed Th1/Th2 responses might involved in balance immune response toward immune activation or cause immunopathogenesis. Both the virulence properties of *L. interrogans* strains and host factors may strongly influence the clinical outcome of the infection.

Our findings increase the knowledge demonstrating that *L. interrogans* possess carbohydrate surface component(s) that can be recognized by DC-SIGN and they are able to activate DCs. Further investigation should be performed such as identification of target host cell surface receptors for more understanding of the molecular mechanism of leptospira pathogenesis and it needed to elucidate the interaction between DCs and *L. interrogans* in detail and to investigate the activation cascade downstream.

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APPENDIX

GENERAL REAGENTS

0.15 M Phosphate-buffered saline (PBS) pH 7.2

NaCl (Fluka)	8.0	gm
KCl (BDH)	0.2	gm
Na_2HPO_4 (Merck)	1.44	gm
KH_2PO_4 (Merck)	0.24	gm

Dissolved in distilled water, adjusted to pH 7.2 with NaOH and adjusted to final volume 1,000 ml with distilled water. Sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches.

TSM buffer

Tris-HCl (Merck)	2.42	gm
NaCl (Merck)	8.76	gm
$CaCl_2$ (Merck)	0.01	gm
$MgCl_2$ (Merck)	0.4	gm

Dissolved in distilled water, adjusted to pH 8.0 with 3 N HCl and adjusedt to final volume 1,000 ml with distilled water.

Blocking buffer (5% bovine serum albumin (BSA) (w/v) in TSMT

TSMT	10.0	ml
BSA	0.5	gm

Mixed well and usually, just prepare before use.

MEDIA FOR CULTURE

Johnson and Seiter (JS) medium

1. Stock solution

NH_4Cl (Merck)	25.0	gm
$ZnSO_4.7H_2O$ (Merck)	0.4	gm
$MgCl_2$ (Merck)	0.695	gm
$CaCl_22H_2O$ (Merck)	1.5	gm
$FeSO_4.7H_2O$ (Merck)	0.5	gm
$CuSO_4.5H_2O$ (Merck)	0.30	gm
Sodium Pyruvate (Merck)	10.0	gm
Thiamine HCl (Merck)	0.50	gm
Cyanocobalamine (Merck)	0.20	gm
Glycerol (Sigma)	10.0	ml
Tween 80 (Merck)	10.0	gm

2. Basal Medium

Na_2HPO_4(anhydrous) (Merck)	1.0	gm
KH_2PO_4 (Merck)	0.30	gm
NaCl (Merck)	1.0	gm

Add the stock solution as follow

NH_4Cl (Merck)	1.0	ml
Thiamine HCl (Merck)	1.0	ml
Sodium Pyruvate (Merck)	1.0	ml
Glycerol (Sigma)	1.0	ml
Distrill water	996	ml

Adjusted the pH to 7.4 and steriled by autoclaving for 15 min at 121°C, 15 lb/square inches.

3. Albumin Supplement

Albumin powder (Sigma)	3.0	gm
$MgCl_2$ (Merck)	300	µl
$ClCl_2$ (Merck)	300	µl
$ZnSO_4$ (Merck)	300	µl

$CuSO_4$ (Merck)	30	µl
$FeSO_4$ (Merck)	3.0	µl
Cyanocobalamine (Merck)	300	µl
Tween 80 (Merck)	3.75	ml

Adjust volume to 30 ml with distilled water. Sterile by filtration using 0.22 µm filter. Usually, just prepare before use.

Luria-Bertani medium (LB medium)

Tryptone (Difco)	10.0	gm
Yeast extract (Difco)	5.0	gm
NaCl (Fluka)	10.0	gm

Dissolve and adjust the volume to 1,000 ml with distilled water. Sterilize by autoclaving for 15 min at 121°C, 15 lb/square inches.

RPMI 1640 complete medium

RPMI 1640 (Gibco)	8.59	ml
100x L-Glutamine (Gibco)	0.1	ml
100x Penicillin/Streptomycin (Gibco)	0.1	ml
100x Sodium pyruvate (Gibco)	0.1	ml
100x MEM Nonessential amino acids (Gibco)	0.1	ml
100x 2-mercaptoetanol (Gibco)	0.01	ml

Penicillin/Streptomycin (1000x)

Streptomycin sulfate	5.0	gm
Penicillin G sodium	$5x10^6$	units
Distilled water	50	ml

Mix well in sterile bottle, filter sterilization (0.2 µm), aliquot and freeze at -20°C.

L-glutamine (100x)

L- glutamine (Gibco)	2.92	gm
Distilled water	100	ml

Mix well in sterile bottle, filter sterilization (0.2 µm), aliquot and freeze at -20°C.

REAGENTS FOR POLYACRELAMIDE GEL ELECTROPHORESIS

Stock solution

10% Sodium dodecyl sulfate (SDS) (w/v)

Sodium dodecyl sulfate (Sigma)	1.0	gm
Distrilled water	10	ml

10% Ammonium persulfate (APS) (w/v)

Ammonium persulfate (Merck)	1.0	gm
Distrilled water	10	ml

0.05% Bromophenol blue (w/v)

Bromophenol blue (Sigma)	0.005	gm
Distrilled water	10	ml

1.5 M Tris-HCL pH 8.8 (resolving gel)

Tris base (USB)	12.0	gm

Dissolve in distrilled water and adjust to pH 8.8 with concentrated HCl and adjust to 100 ml final volume.

1.0 M Tris-HCL pH 6.8 (stacking gel)

Tris base (USB)	12.0	gm

Dissolve in distrilled water and adjust to pH 6.8 with concentrated HCl and adjust to 100 ml final volume.

10% SDS (w/v)

SDS (Sigma)	1.0	gm
Distrilled water	10	ml

12% Resolving gel

Distrilled water	1.6	ml
30% Acrelamide mix	2.1	ml
1.5 M Tris-HCl pH.8.8	1.3	ml
10% SDS	50.0	µl
10% APS	50.0	µl
TEMED (Promega)	2.0	µl

3.5% Stacking gel

Distrilled water	1.4	ml
30% Acrelamide mix	330.0	µl
1.5 M Tris-HCl pH.8.8	250.0	µl
10% SDS	20.0	µl
10% APS	20.0	µl
TEMED (Promega)	2.0	µl

Staining solution

Coomassie brillian blue R250 (Fluka)	1.0	gm
Methanol (J.T.Baker)	152	ml
Glacial acetic acid (BHD)	35	ml

Dissolve and adjust the volume to 500 ml with distrilled water.

Destaing solution

Methanol (J.T. Baker)	500	ml
Glacial acetic acid (BHD)	140	ml

Add distrilled water to final volume 2,000 ml.

BIOGRAPHY

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