

Hamster IFN- γ ⁺CD4⁺ and IL-4⁺CD4⁺ T cell responses against leptospire are significantly higher than those of mice

Yaowarin Nakornpakdee,^{1,3} Rasana W Sermswan,^{2,3} Santi Maneewatcharangsri,⁴ Surasakdi Wongratanacheewin,^{1,3}

Abstract

Background: Leptospirosis is a bacterial disease caused by the *Leptospira interrogans*. The hamster is considered a susceptible host while the mouse is resistant. The knowledge of hamster T cell immunity is limited compared to the mouse. The reason why the hamster and the mouse give different responses to leptospire remains unclear.

Objective: To determine the differential responses of CD4⁺ T cells between hamsters and mice using *Leptospira interrogans* as an infectious model.

Methods: The CD4⁺ T-cell reactivity and their intracellular cytokine responses after infection with live *L.interrogans* serovar Autumnalis or leptospiral antigens, or injection with recombinant LipL32 protein (rLipL32) were elucidated. For secondary immune responses, mononuclear cells were re-stimulated with leptospiral crude antigens (LAg) or rLipL32. Intracellular cytokines and CD4⁺ T cells were determined using flow cytometry.

Results: There were no significant differences between the percentages of hamster and mouse CD4⁺ and CD25⁺CD4⁺ T cell responses to live bacteria. Mouse CD4⁺ (24.50±1.98%) and CD25⁺CD4⁺ T cells (3.83±0.88) responded significantly higher than those of hamster (15.07±2.82% and 2.00±0.37%) when infected and re-stimulated with LAg. The numbers of IFN- γ and IL-4 producing cells in hamsters at 1.76±0.10% and 0.82±0.25% for IFN- γ ⁺CD4⁺ and IL-4⁺CD4⁺ T cells were significantly higher than those in resistant mice at 0.10±0.02% and 0.23±0.03% for IFN- γ ⁺CD4⁺ and IL-4⁺CD4⁺ T cells.

Conclusion: Hamsters responded significantly higher in secondary stimulation especially in the levels of the IFN- γ ⁺ and IL-4⁺CD4⁺ T cells. The mechanisms of this dissimilarity remain to be elucidated.

Keywords: Leptospirosis, LipL32, *L.interrogans* serovar Autumnalis, T cell response, CD4

From:

¹ Department of Microbiology,

² Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand

³ Melioidosis Research Center, Khon Kaen University, Khon Kaen, 40002, Thailand

⁴ Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand

Corresponding author:

Surasakdi Wongratanacheewin

Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand

Email: sura_wng@kku.ac.th

Introduction

Leptospirosis is a worldwide zoonotic disease caused by the pathogenic *Leptospira* genus and there is variable host susceptibility toward pathogenic *Leptospira* strains. The commonly used animal models for leptospirosis studies are hamsters and guinea pigs while mice and rats are generally resistant to leptospirosis and are often found to be reservoirs of the bacteria.¹ Although the clinical aspects and progression of the disease are well understood, knowledge of host factors which determine the outcome of infection is limited.² As the hamsters and mice give different responses to leptospire

especially in pathogenesis and protection, the variances in these models were therefore studied. The animal models with different susceptibilities to leptospire may help discovering the crucial factors for survival of the infection. The Syrian hamster is highly susceptible to many organisms and has been used as an excellent experimental model for several infectious diseases caused by microorganisms, such as *Treponema pallidum*,³ *Leishmania* spp.,⁴ *Opisthorchis viverrini*,⁵ and *Leptospira interrogans*.⁶ It is still unclear, however, why the hamster is extremely susceptible to such infections

and gives different outcomes to leptospirosis compared to the well characterized mouse model. In the mouse model, the interaction between host and pathogens can induce chemokine expression and different levels of host susceptibility can give differential chemokine profiles. BALB/c mice are considered as the most resistant mouse model against leptospirosis and gave the highest level of chemokine expression compared to C3H/HeJ and C3H/HePas which are sensitive and have intermediate susceptibility.⁷ Due to the highest resistance level in BALB/c mouse, it was accordingly selected as the model to compare with hamsters which are susceptible to this pathogen. Furthermore, previous studies reported that antigen-presenting cells (APCs) present the processed leptospiral antigens to CD4⁺ T cells through MHC Class II molecules, leading to their activation and production of cytokines such as IL-4 and IFN- γ to support the role of B cells in protection against leptospires.^{8,9} Therefore, the responses of CD4⁺ T-cell subsets and their intracellular cytokines, IFN- γ and IL-4, were studied between susceptible hamsters and resistant BALB/c mice infected with virulent *L.interrogans* serovar Autumnalis or the recombinant LipL32 protein (rLipL32) and were compared by flow cytometry in this study. In addition, the differences in responses of mice and hamsters to *L.interrogans* might also reflect the dissimilarities between hamster and mouse immunities.

Methods

Animals and ethics

Outbred 4 week old female Syrian golden hamsters obtained from the Animal Laboratory Breeding Unit, Faculty of Medicine, Khon Kaen University and four-week-old inbred female BALB/c mice purchased from Nomura Siam International Co. Ltd. were used in this study. All animals were maintained in the animal care unit at Faculty of Medicine, Khon Kaen University. All experiments were approved by the Animal Ethics Committee of Khon Kaen University (No. AEK KU 6/2558 and AEKKU-NELAC 3/2558, No. 0514.1.75/1) and performed in accordance with institutional guidelines.

Antigen Preparation

1. Leptospiral crude antigens (LAG)

LAG was prepared as described.¹⁰ Briefly, *L.interrogans* serovar Autumnalis UI13372 was cultured in *Leptospira* medium Ellinghausen–McCullough–Johnson–Harris (EMJH) (Becton, Dickinson and Company, Maryland, USA) at 30 °C for 7-10 days to yield a cell density of 10⁸ cells/ml. Bacteria were harvested by centrifugation at 10,000 \times g for 10 minutes and killed with 0.5 mg/l sodium azide for 30 minutes. The bacteria were washed twice in 0.01 M phosphate-buffered saline (PBS), pH 7.4, resuspended in PBS, and frozen at -20 °C for 7 days. They were centrifuged at 10,000 \times g for 30 minutes at 4 °C after being thawed. The pellets were washed two times with PBS, resuspended in PBS, and sonicated on ice at 20 kHz (High intensity ultrasonic processor model VC/VCX 750, Sonics) for 3 periods of 3 minutes each.

LAG was filtered with 0.2 μ m pore size filter membranes (Whatman, Buckinghamshire, England) and the protein concentrations were determined using Bradford reagents (Bio-rad,

CA, USA). The sterility of proteins was confirmed by absence of bacterial growth on Luria Bertani (LB) agar plates at 37°C and EMJH media at 30°C. The contaminated endotoxins were determined by the *Limulus ameobocyte* lysate (LAL) assay using Pierce LAL Chromogenic Endotoxin Quantitation Kits (Thermo Fisher Scientific, MA, USA). The proteins were kept at -20 °C until used.

2. Recombinant LipL32 protein (rLipL32)

rLipL32 was produced from BL21(DE3) *E.coli* carrying the recombinant *lipl32*-pET23a(+) plasmid as described previously with modifications.^{11,12} Briefly, the transformed *E.coli* was grown in LB with 100 μ g/ml ampicillin (LB-A) at 37 °C with shaking at 200 rpm. The rLipL32 protein expression was induced by 0.2 mM IPTG at 37 °C for 3 hours.

The His6-tagged rLipL32 was purified from crude solubilized protein prepared from bacterial inclusion bodies by a Ni-NTA affinity column (GE Healthcare, Uppsala, Sweden) under a denaturing condition. The rLipL32 was concentrated and its buffer was exchanged to RPMI1640 plain medium (Gibco, Thermo Fisher Scientific, MA, USA) using a 3 kDa cut-off Amicon Ultra-tubes (Merck Millipore, County Cork, Ireland) at 4 °C, followed by filtration with a 0.2 μ m filter membrane (Whatman, Buckinghamshire, England). Protein concentration was measured by a BCA protein assay kit (Thermo Fisher Scientific, MA, USA) and the aliquots of proteins were stored at -20 °C.

The rLipL32 protein was analyzed by reverse phase nano-liquid chromatography (Dionex, Surrey, UK) coupled with MicroToF Q II mass spectrometry (Bruker, Bremen, Germany) and the mass spectrometric result was identified using the MASCOT search engine 2.2 (Matrix Science, Ltd.). Protein purity of rLipL32 protein was verified under 13% SDS-PAGE and colloidal Coomassie Brilliant Blue G-250 stain. Antigenic specificity of rLipL32 was confirmed by Western blotting using anti-6x His antibody (“R&D Systems, MN, USA”). The protein sterility and contaminated endotoxins in the rLipL32 were determined as the same in LAG preparation.

Leptospira infection and rLipL32 injection

1. Live *L.interrogans* serovar Autumnalis infection

Hamsters and BALB/c mice were divided into three groups, 3 per group, including a non-injected group as a normal control, an EMJH-injected, and a 10² live *L.interrogans* serovar Autumnalis-infected group. Hamsters and BALB/c mice were injected intraperitoneally with EMJH or 10² live *L.interrogans* serovar Autumnalis on day 0. After 10 days of infection, all animals were sacrificed and spleens were collected.

2. rLipL32 injection

Hamsters and BALB/c mice were divided into four groups, 3 per group, including a non-injected group as a normal control, an RPMI1640-injected, a TiterMax gold adjuvant (Sigma-Aldrich, USA) injected group and a 20 μ g of rLipL32 emulsified in adjuvant-injected group. Hamsters and BALB/c mice were injected intraperitoneally with RPMI1640, TiterMax gold adjuvant, or rLipL32 on day 0. The same antigens were subcutaneously injected at multiple sites on the backs of the hamsters and BALB/c mice on days 7, 14, and 21. Spleens were

collected 3 days after the last injection.

Flow cytometric analysis

Fluorescent antibodies

Anti-mouse antibodies used in this study were CD4-PE/Cy7 (GK1.5), CD25-Pacific blue (PC61), IFN- γ -FITC (XMG 1.2), and IL-4-PerCP/Cy5.5 (11B11). Isotypic controls were rat IgG2b-PE/Cy7 (RTK4530), rat IgG1-Pacific blue (RTK2071), rat IgG1-FITC (RTK2071), and rat IgG1-PerCP/Cy5.5 (RTK 2071). All antibodies were purchased from Biolegend.

Cell stimulation and surface immunofluorescence staining

Splenic mononuclear cells were isolated from all experimental animals with Ficoll-Paque solution (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocols. Splenic mononuclear cells were sequentially stained with fluorescent antibodies and analyzed by flow cytometry. Besides flow cytometric analysis, hamster and mouse splenic mononuclear cells derived from live serovar Autumnalis infections were stimulated with RPMI1640 alone as an unstimulated control or with 20 μ g/ml of LAg at 24 and 48 hours. Those derived from rLipL32 injections were stimulated with RPMI1640 alone as an unstimulated control or 20 μ g of rLipL32 at 48 hours. In brief, splenic mononuclear cells were suspended in complete RPMI1640 medium (RPMI1640 medium supplemented with 10% FBS, 100 U/mL Penicillin and 100 μ g/mL Streptomycin). One million cells were cultured with or without mentioned antigens in 48-well plates and 5 μ g/ml of Brefeldin A (Biolegend, CA, USA) as a protein transport inhibitor was added into the cultures 12 hours before harvesting. The stimulated cells were centrifuged at

350 \times g for 5 minutes at 4 $^{\circ}$ C. The culture cells were then washed in fluorescence-activated cell sorting (FACS) staining buffer (PBS, 5% FBS, and 0.1% sodium azide) and resuspended in 50 μ l of FACS staining buffer containing an optimal concentration of the desired fluorescent antibodies and 2% of normal rat serum as Fc receptor blocking. Cells were washed with FACS staining buffer after being incubated for 30 minutes at room temperature. Intracellular staining was subsequently performed.

Intracellular cytokine staining

To analyze intracellular cytokine production, the cell surface marker stained cells were fixed with Cytofix/Cytoperm solution (BD Biosciences, CA, USA) for 20 minutes at 4 $^{\circ}$ C and washed twice with Perm/Wash solution (BD Biosciences, CA, USA). Fixed and permeabilized cells were thoroughly resuspended in 50 μ l of Perm/Wash solution containing an optimal concentration of anti-IFN- γ -FITC (XMG1.2), anti-IL-4-PerCP/Cy5.5 (11B11), or isotype control antibody (Biolegend, CA, USA) and incubated at 4 $^{\circ}$ C for 1 hour in the dark. The cells were then washed twice with Perm/Wash solution and resuspended in FACS staining buffer prior to flow cytometric analysis.

All samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences, CA, USA) and data were analyzed by FlowJo software 10.2 (FlowJo LLC, OR, USA). Lymphocytes were gated based on an FSC-SSC gate. The stained anti-CD4 mAb areas were subsequently gated and defined as percentages of CD4⁺ T cells of lymphocytes. Further gating adjustments were performed based on the expressions of CD25, IFN- γ , and IL-4 (**Figure S1-S4**). Percentages of each cell subpopulation were calculated. Isotype controls of each antibody were included in each staining protocol.

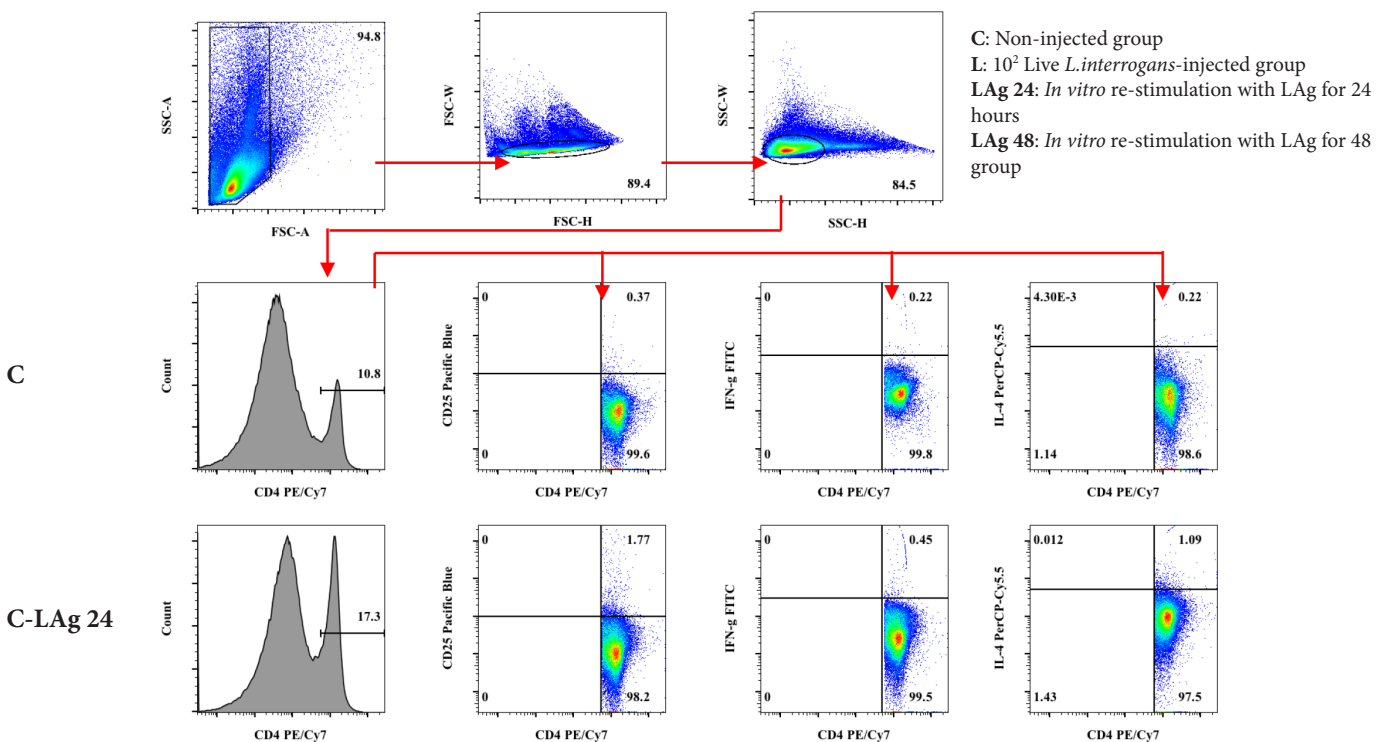


Figure S1. Flow cytometry gating strategy. Hamster cells derived from live *L.interrogans* serovar Autumnalis infection and LAg re-stimulation for 24 and 48 hours were stained with indicated antibodies and examined by flow cytometry. FSC-W, FSC-H, SSC-W and SSC-H were used to gate out doublet cells after applying the FSC-SSC gate. After selection for CD4⁺ cells, further gating adjustments were performed based on the expressions of CD25, IFN- γ , and IL-4.

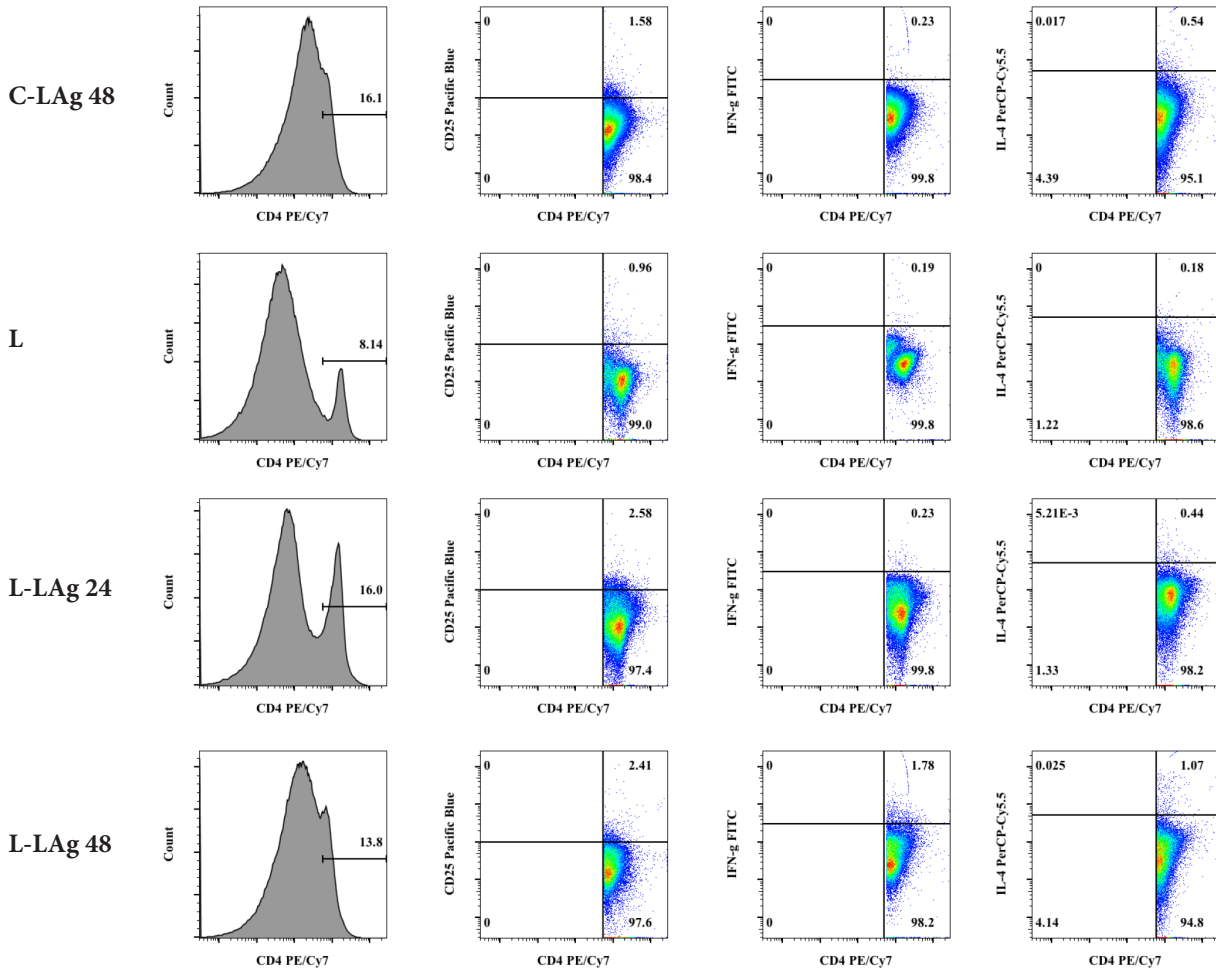


Figure S1. (Continued) Flow cytometry gating strategy.

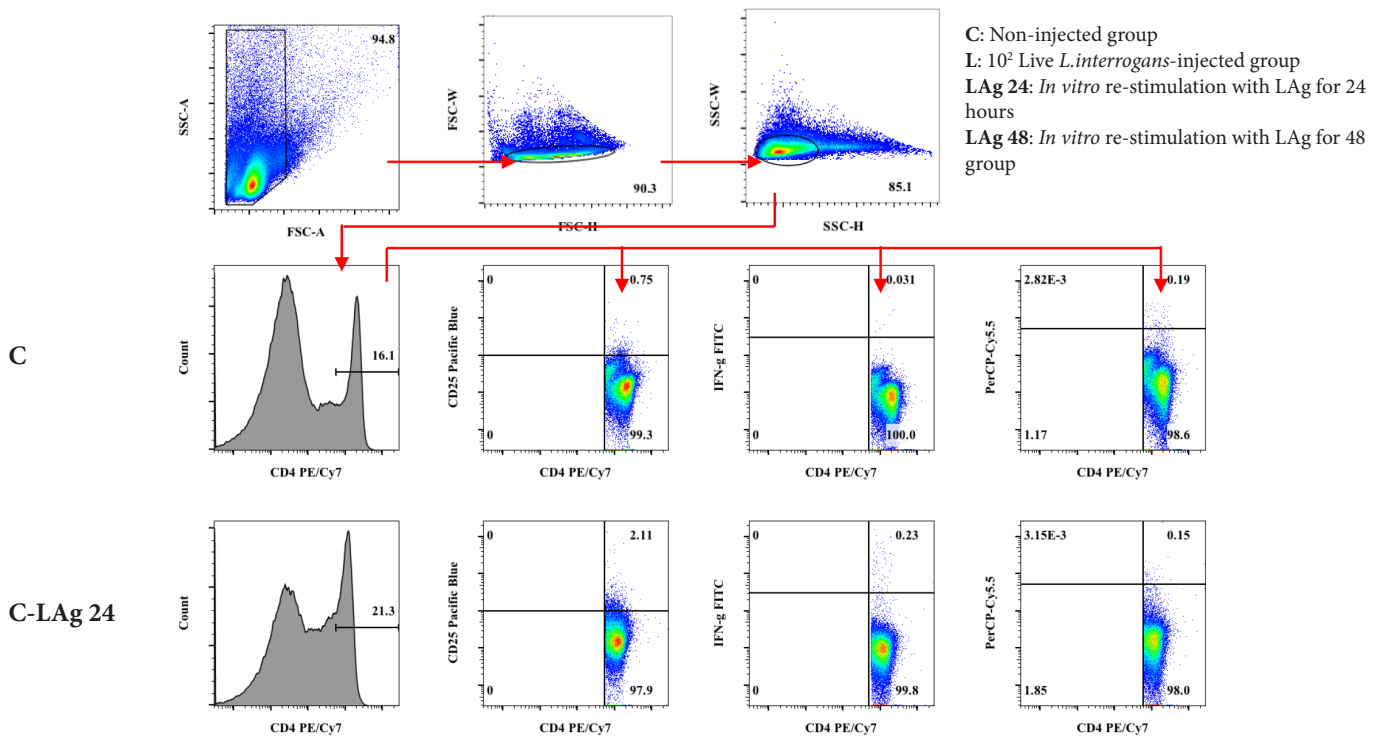


Figure S2. Flow cytometry gating strategy. Mouse cells derived from live *L.interrogans* serovar Autumnalis infection and LAg re-stimulation for 24 and 48 hours were stained with indicated antibodies and examined by flow cytometry. FSC-W, FSC-H, SSC-W and SSC-H were used to gate out doublet cells after applying the FSC-SSC gate. After selection for CD4⁺ cells, further gating adjustments were performed based on the expressions of CD25, IFN- γ , and IL-4.

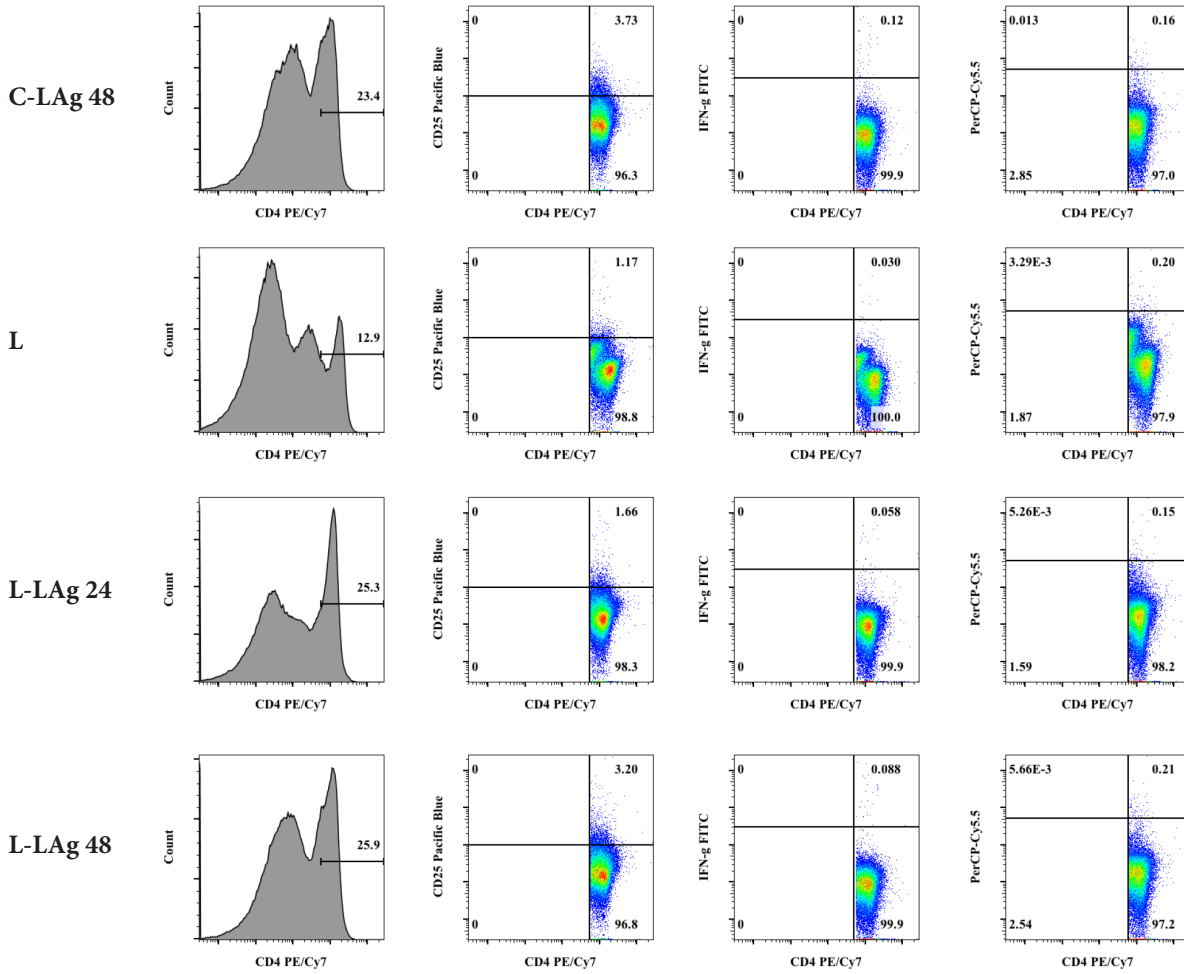


Figure S2. (Continued) Flow cytometry gating strategy.

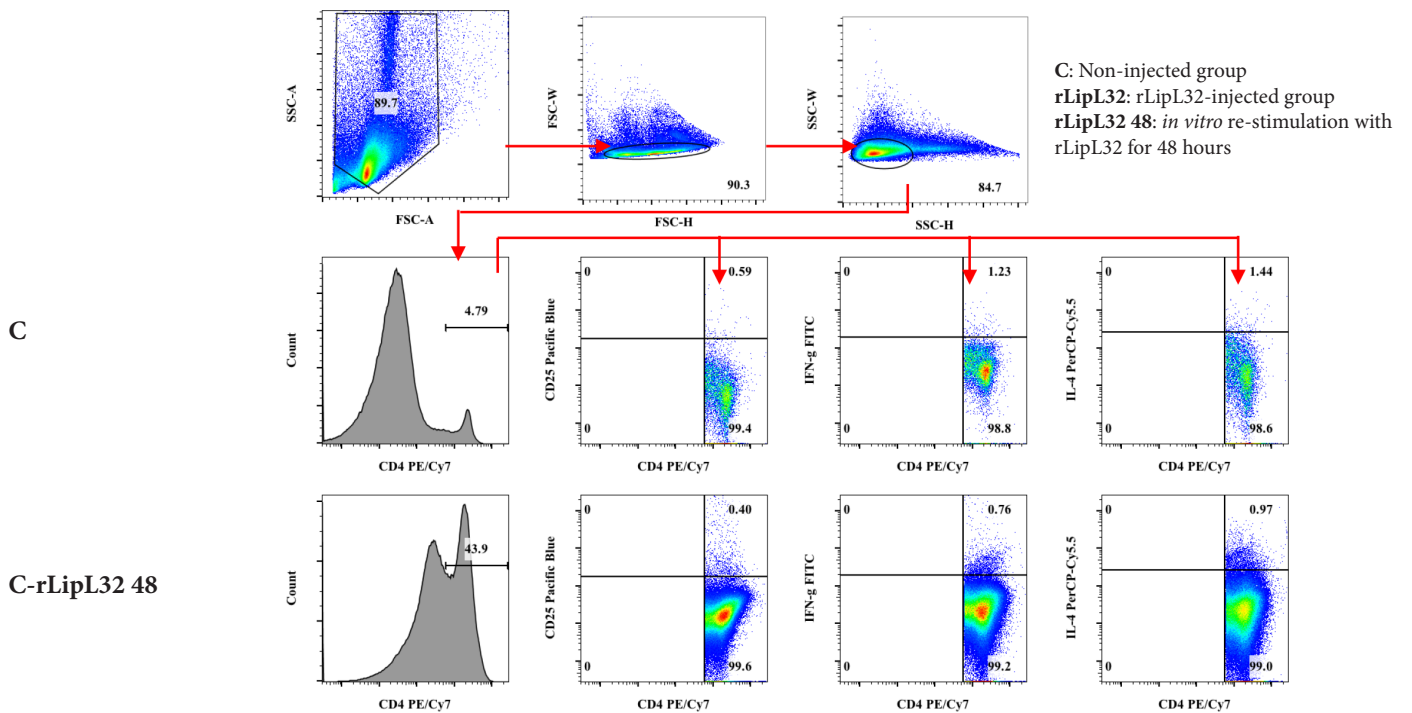


Figure S3. Flow cytometry gating strategy. Hamster cells derived from rLipL32 infection and rLipL32 re-stimulation for 48 hours were stained with indicated antibodies and examined by flow cytometry. FSC-W, FSC-H, SSC-W and SSC-H were used to gate out doublet cells after applying the FSC-SSC gate. After selection for CD4⁺ cells, further gating adjustments were performed based on the expressions of CD25, IFN- γ , and IL-4.

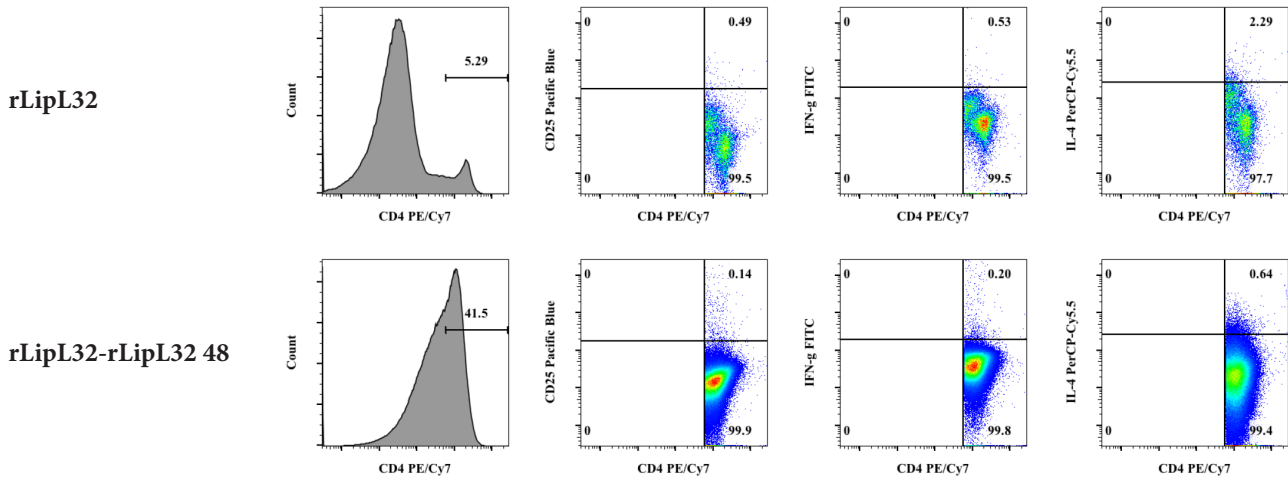


Figure S3. (Continued) Flow cytometry gating strategy.

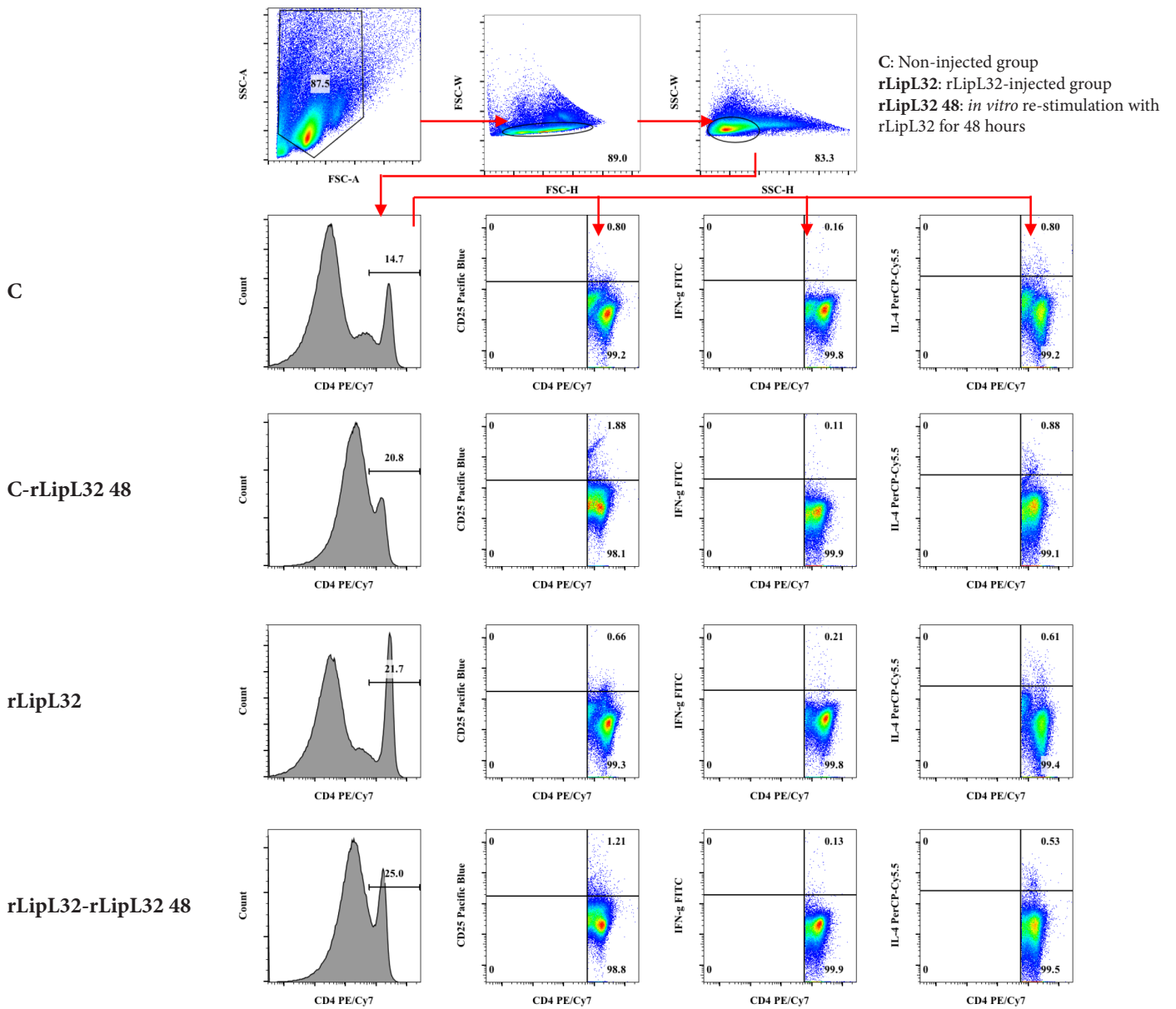


Figure S4. Flow cytometry gating strategy. Mouse cells derived from rLipL32 infection and rLipL32 re-stimulation for 48 hours were stained with indicated antibodies and examined by flow cytometry. FSC-W, FSC-H, SSC-W and SSC-H were used to gate out doublet cells after applying the FSC-SSC gate. After selection for CD4⁺ cells, further gating adjustments were performed based on the expressions of CD25, IFN- γ , and IL-4.

Statistical analysis

Data are shown as means ± standard deviations (SDs). The one-way ANOVA was used to analyze multiple groups and Student's *t*-test was used to compare data between mouse and hamster in each parameter. The comparison data with *P* values <0.05 were considered as statistically significant differences.

Results

Mouse CD4⁺ and CD25⁺CD4⁺ T cells respond significantly higher than those of the hamster when infected with *L. interrogans*

Hamster and mouse T cell subsets that responded to *L. interrogans* infection were investigated using commercially available anti-mouse antibodies (Table 1) which had previously been tested for cross-reactivity in the Syrian golden hamster. The CD4⁺ and CD25⁺CD4⁺ T cells derived from spleens of

hamsters and BALB/c mice with or without live *L. interrogans* serovar Autumnalis infection were identified by flow cytometry. There were no significant differences between percentages of hamster and mouse CD4⁺ and CD25⁺CD4⁺ T cells responding to live bacteria in both *L. interrogans* infected and control groups although mouse CD4⁺ T cells were slightly increased in both infected and control groups. The %CD4⁺ T cells in the mouse model under *in vitro* LAG re-stimulated conditions at 48 hours were significantly higher than those of the hamster. This phenomenon was also found in CD25⁺CD4⁺ T cells except that the responses of hamster CD25⁺CD4⁺ T cells at 24 hours were significantly greater than in the mouse. In addition, *in vitro* LAG re-stimulation exhibited higher responses of CD4⁺ and CD25⁺CD4⁺ T cells compared to conditions without re-stimulation (Figure 1A-B). The overall CD4⁺ and CD25⁺CD4⁺ T cell responses in the mouse were significantly higher than in the hamster.

Table 1. Antibodies used in this study

Antigens	Clones	Host species	Reactivity	Isotype	References
CD4	GK1.5	Rat	Mouse	IgG2b	Dondji et al., 2008; Hammerbeck et al., 2011
CD25	PC61	Rat	Mouse	IgG1	Kaewraemruaen et al., 2016
IFN- γ	XMG1.2	Rat	Mouse	IgG1	Kaewraemruaen et al., 2016
IL-4	11B11	Rat	Mouse	IgG1	Kaewraemruaen et al., 2016

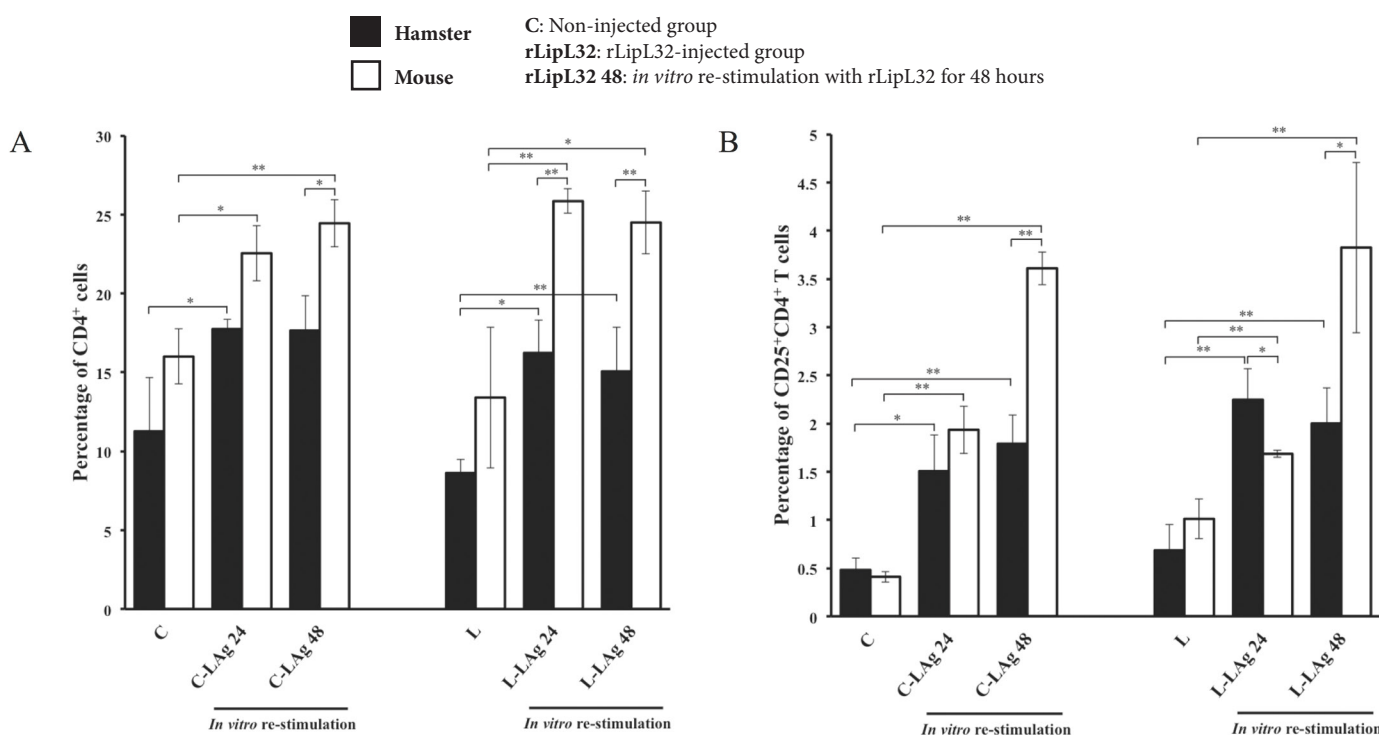


Figure 1. Quantification of CD4⁺ T cells (A) CD25⁺CD4⁺ T cells (B) IFN- γ ⁺CD4⁺ T cells (C) and IL-4⁺CD4⁺ T cells (D) derived from *Leptospira* infection. Hamsters and BALB/c mice were intraperitoneally injected with EMJH or 10² live *L. interrogans* serovar Autumnalis on day 0. The non-injected group served as a control. Spleens were collected after 10 days of infection and splenic mononuclear cells were isolated. Splenic mononuclear cells were analyzed by flow cytometry and also cultured with or without 20 μ g/ml of LAG for 24 and 48 hours. Cells were stained with anti-mouse (CD4, CD25, IFN- γ , and IL-4) mAbs. Data are reported as means ± standard deviations for three animals per group. Statistically significant differences were evaluated using one-way ANOVA and Student's *t*-test. The asterisks (*) and (**) indicate statistical significance at *p*<0.05 and *p*<0.01 when compared with controls.

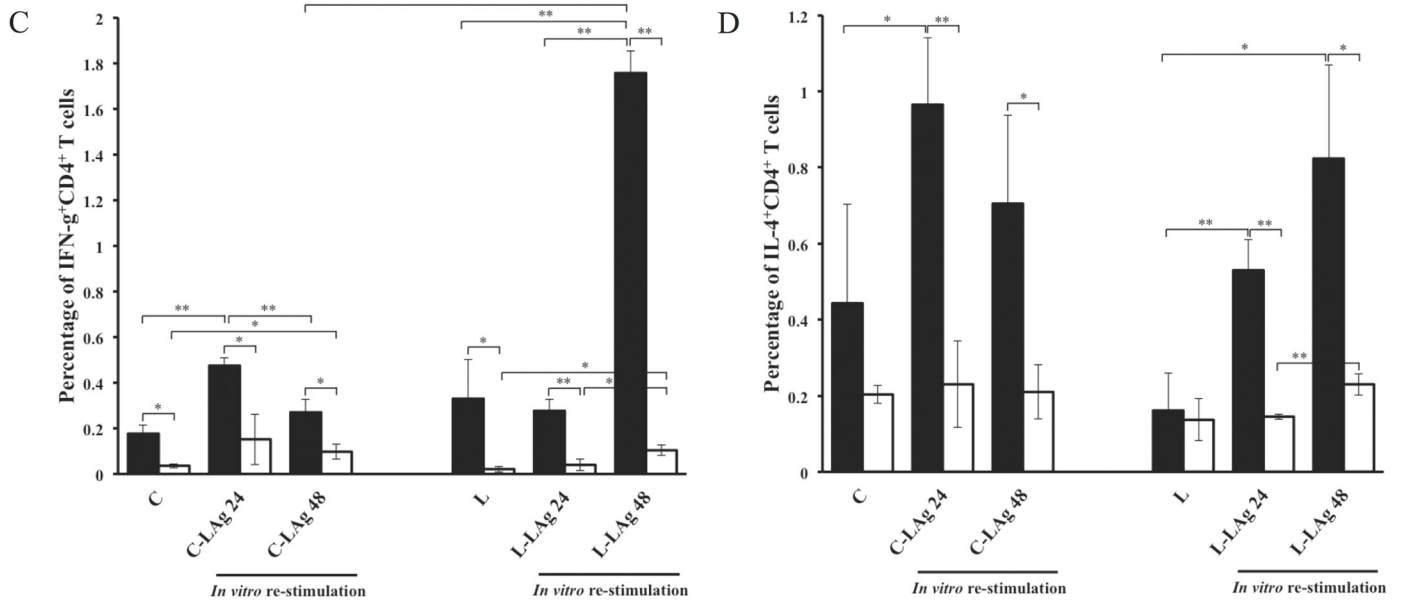


Figure 1. (Continued) Quantification of CD4⁺ T cells (A) CD25⁺CD4⁺ T cells (B) IFN- γ ⁺CD4⁺ T cells (C) and IL-4⁺CD4⁺ T cells (D) derived from *Leptospira* infection.

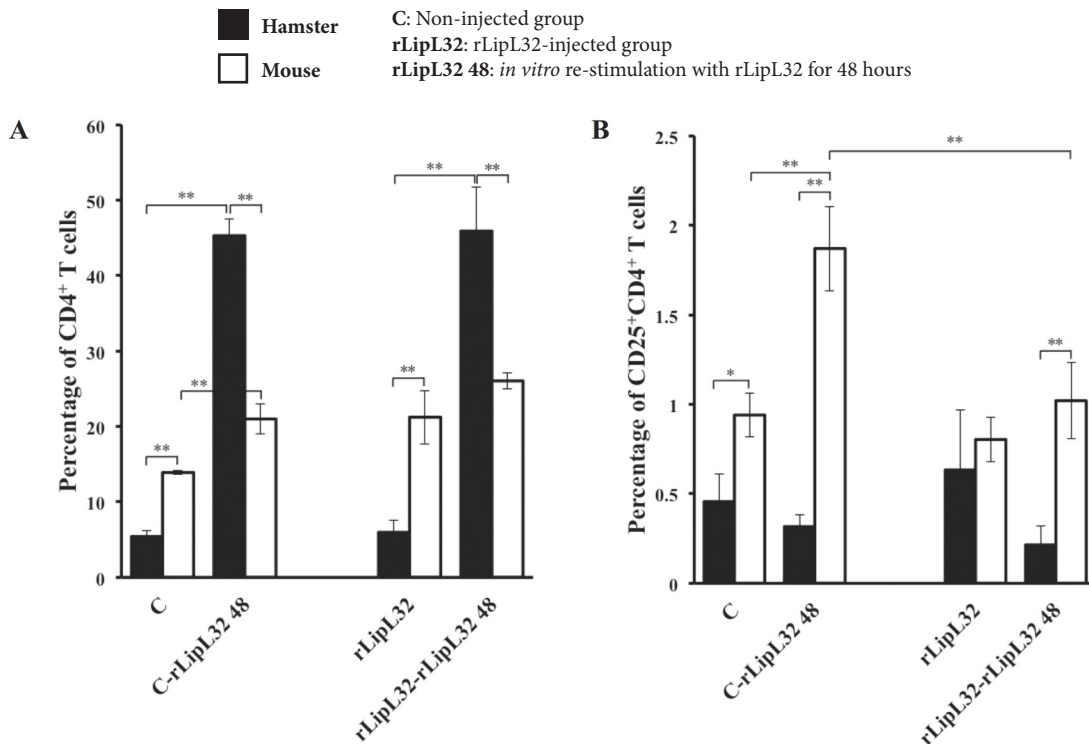


Figure 2. Quantification of CD4⁺ T cells (A) CD25⁺CD4⁺ T cells (B) IFN- γ ⁺CD4⁺ T cells (C) and IL-4⁺CD4⁺ T cells (D) derived from rLipL32 injection. Hamsters and BALB/c mice were intraperitoneally injected with RPMI1640, Adjuvant (TiterMax gold adjuvant), or 20 μ g of rLipL32 on day 0. The non-injected group served as a control. The same antigens were subcutaneously injected at multiple sites on the backs of hamsters and BALB/c mice on days 7, 14, and 21. Splens were collected 3 days after the last injection and splenic mononuclear cells were isolated. Splenic mononuclear cells were analyzed by flow cytometry and also cultured with or without 20 μ g/ml of rLipL32 for 48 hours. Cells were stained with anti-mouse (CD4, CD25, IFN- γ , and IL-4) mAbs. Data are reported as means \pm standard deviations for three animals per group. Statistically significant differences were evaluated using one-way ANOVA and Student's *t*-test. The asterisks (*) and (**) indicate statistical significance at $p < 0.05$ and $p < 0.01$ when compared with controls.

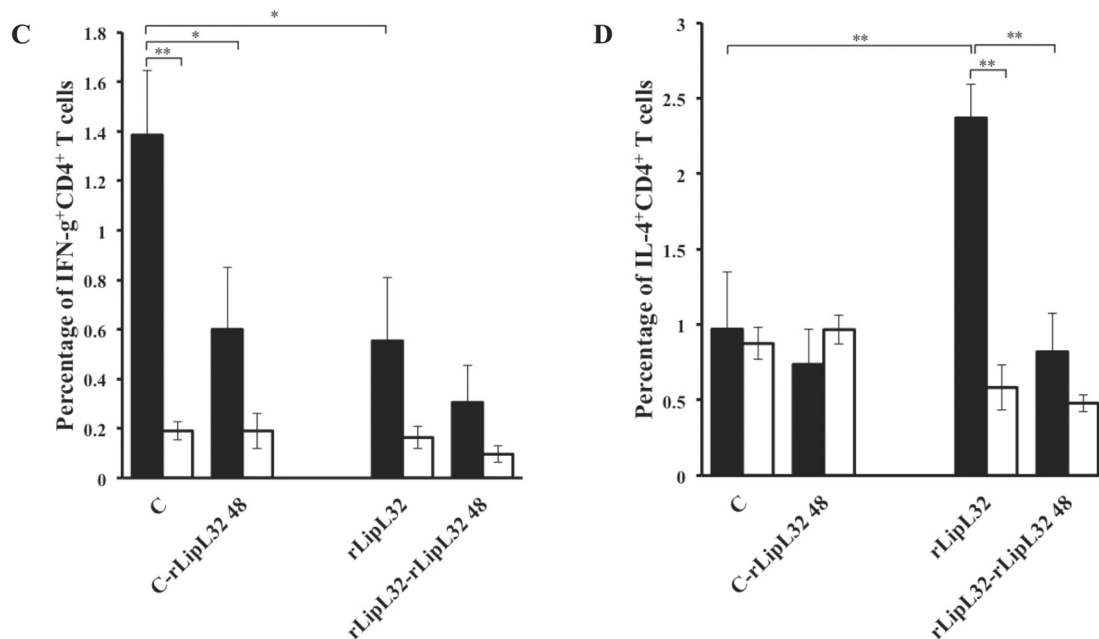


Figure 2. (Continued) Quantification of CD4⁺ T cells (A) CD25⁺CD4⁺ T cells (B) IFN- γ ⁺CD4⁺ T cells (C) and IL-4⁺CD4⁺ T cells (D) derived from rLipL32 injection.

The IFN- γ ⁺CD4⁺ and IL-4⁺CD4⁺ T cells of hamsters responded significantly higher than those of mice

In contrast to the percentage of CD4⁺ T cells, the percentages of IFN- γ ⁺CD4⁺ and IL-4⁺CD4⁺ T cells in hamsters were significantly higher than those in mice among all groups. *L.interrogans* serovar Autumnalis-infected hamsters with LAg re-stimulation for 48 hours gave the strongest response of IFN- γ ⁺CD4⁺ T cells (1.76 \pm 0.10%). *In vitro* LAg re-stimulation conditions showed significantly higher responses of IL-4⁺CD4⁺ T cells than conditions without LAg re-stimulation. This circumstance, however, occurred only in hamsters pre-infected with live *L.interrogans* (Figure 1C-D). This might indicate the hamster CD4⁺ T cells produced either IFN- γ or IL-4 differently from mice.

Hamster CD4⁺ T cells responded against rLipL32 differently from the mice

As LipL32 is the common surface protein of pathogenic leptospire serovars, it was then used as the stimulation antigens in this study for the investigation of differential responses between hamsters and mice. Similar to live infections, the numbers of mouse CD4⁺ T cells (21.23 \pm 3.55%) were significantly higher than the hamsters (5.98 \pm 1.59%). Interestingly, after *in vitro* re-stimulation with rLipL32, hamster CD4⁺ T cells (45.90 \pm 5.80%) were significantly greater than mouse cells (26.05 \pm 1.06%) while the hamster CD25⁺CD4⁺ T cells (0.22 \pm 0.11%) were significantly lower than mouse cells (1.02 \pm 0.21%) (Figure 2A-B). While the IFN- γ ⁺CD4⁺ and IL-4⁺CD4⁺ T cells of both animals were comparable; LipL32 stimulated slightly higher, but not significantly, hamster IFN- γ ⁺CD4⁺ T cells (0.55 \pm 0.26% and 0.31 \pm 0.15% for LipL32 injections and injections with *in vitro* re-stimulation) than in the mouse (0.16 \pm 0.05% and 0.10 \pm 0.03% for LipL32 injections and injections with *in vitro* re-stimulation) (Figure 2C). In contrast, the hamster gave a significantly higher number of

IL-4⁺CD4⁺ T cells than those of the mouse in the primary response (Figure 2D). This result indicates the striking difference of both animal models to the common pathogenic leptospiral antigens. These data suggested that T cell responses elicited against *Leptospira* protein, LipL32, are different between hamster and mouse models.

Discussion

The humoral-mediated immune response is known to be a major immune system component against leptospirosis as leptospires are extracellular pathogens¹³ while the knowledge of the T cell response to this disease remains poorly understood. Several animal models have been used to elucidate host immune responses and leptospirosis pathology. Hamsters, guinea pigs, and gerbils are susceptible to leptospirosis while mice and rats are resistant.¹ In order to discover the crucial factors for the host defense mechanisms in survival to leptospirosis and provide more strategies to control this disease, *Leptospira*-specific CD4⁺ T-cell subsets and the cytokine release associated with different host susceptibilities to leptospires were analyzed between susceptible hamsters and resistant BALB/c mice. Although the Syrian hamster is highly susceptible to many organisms and has been used as an excellent experimental model for several infectious diseases, it remains unclear why the hamster is extremely susceptible to such infections and gives the different outcomes in leptospirosis compared to the well characterized mouse model. It might be because of limited availability of immunological reagents, specific monoclonal antibodies (mAbs), and molecular tools to study the immune system of this hamster model. The production and development of new specific mAbs is time-consuming and expensive. Several commercially available anti-mouse mAbs including anti-mouse CD4 clone GK1.5,¹⁴ anti-rat CD8 β clone 341,¹⁵ anti-mouse CD25 clone PC61, anti-mouse IFN- γ clone XMG1.2, and IL-4 clone 11B11⁵ are available which have previously been shown to

cross-react with hamsters and were thus used to determine the responses of CD4⁺ T-cell subsets and their intracellular cytokines, IFN- γ and IL-4, between leptospirosis susceptible hamsters and resistant BALB/c mice in this study. Although the outbred hamsters were used to compare with the inbred mice in this study, most of the available hamsters were extensively line bred from the same mother and litters so that they could be closely related to inbred stock. Inbred hamsters are usually unhealthy with shorter life spans than those constantly outcrossed. Thus, a limitation regarding this point could not be excluded. The severity of outcomes in leptospirosis has been based considerably on the environment, pathogen virulence, and host susceptibility.¹⁶ Host immune responses are hypothesized to be the more significant ones to exhibit the dramatic symptoms of the disease than virulence of the pathogen.¹ In this study, the *ex vivo* phenotypes of CD4⁺ T-cell subsets were compared among different groups. The results demonstrated that there were no differences between the hamster or mouse models. This indicates similar CD4⁺ T-cell stimulation of leptospiral antigens in both animals. After *in vitro* re-stimulation with rLipL32 for 48 hours, the responses of the mouse CD4⁺ and CD4⁺CD25⁺ T cells were significantly higher than those of the hamster. This might be due to different secondary immune responses leading to the more rapid production of chemokines which are important for recruitment and activation of T cells in the resistant model compared to susceptible models. Several studies compared the immune responses of the host with different susceptibilities to leptospires. The immune responses of the susceptible Syrian golden hamster were compared with the resistant Oncins France 1 (OF1) mouse in terms of histological analysis, cytokine mRNA expression, and the quantification of leptospire loads in target organs and blood. Severe outcomes such as hemorrhage, inflammation, and augmentation of leptospire burdens were found in hamster organs, while a rapid clearance was observed in the mice resulting in limited changes in histological observations. The pro-inflammatory cytokines TNF- α , IL-1 β , cyclo-oxygenase-2, and IL-6 and anti-inflammatory cytokine IL-10 were delayed and vast overexpression in the hamster occurred while rapid induction was found in mice. The same result was also observed for the chemokines, IP-10/CXCL10 and MIP-1 α /CCL3. The rapid cytokine production and recruitment of immune cells, especially T cells, in resistant mice might be the important factor to rapidly controlling leptospires and limiting pathological lesions.¹⁷ Although the numbers of mouse CD4⁺ T cells was higher than those of hamster CD4⁺ T cells, these cells produced low levels of IFN- γ and IL-4. The high production of hamster IFN- γ ⁺CD4⁺ T cells may lead to the marked inflammation of infected hamsters causing animal death. This finding was also reported in previous data by the present authors¹⁸ when heat-killed vaccine protected hamsters from leptospirosis with lower levels of IFN- γ ⁺CD4⁺ hamster cells. Another explanation might be due to the various subpopulations of CD4⁺ T cells with distinct cytokine profiles between hamsters and mice giving the different responses. As the antibody used for determination of the number of hamster CD4⁺ and CD4⁺CD25⁺ T cells were anti-mouse antibodies, therefore, the low reactivity to hamster cells could not be excluded. The *L.interrogans* serovar

Autumnalis-injected hamsters with LAg re-stimulation for 48 hours gave the greatest response of IFN- γ ⁺CD4⁺ T cells among all samples (**Figure 1C**). This indicates that the primary infection with *L.interrogans* serovar Autumnalis primes the populations of antigen-specific hamster CD4⁺ T cells resulting in the high level of IFN- γ production when re-stimulated with LAg correlated with host susceptibilities to this infection. The study of specific CD4⁺ T cell reactivity in various clinical outcomes of leptospirosis patients reported that the response of IFN- γ ⁺CD40L⁺CD4⁺ T cells derived from whole-blood specimens stimulated with the leptospiral antigen *in vitro* and was correlated with the severity of leptospirosis in these patients.⁹

LipL32 is derived only from pathogenic strains of *Leptospira* and is a well-known outer membrane protein.¹⁹ According to this previous study, the *in vivo* gene expression of *Leptospira* LipL32 was quantified in blood of animal models with different susceptibilities to leptospires; the susceptible Syrian golden hamster and the resistant BALB/c mouse. Their results indicated that the *lipl32* expression in hamsters was significantly higher than in mice.²⁰ This result may correlate with the present data in which the responses of hamster LipL32-specific CD4⁺ T cells were higher than those of the mouse model. Although the response of hamster CD4⁺ T cells was dramatically increased in *in vitro* re-stimulation with rLipL32, lower levels of IFN- γ and IL-4 producing CD4⁺ T cells were detected. This might be because of the different stimulations of epitopes in mice and hamsters.

Conclusion

Taken together, the results of the present study appear to be the first report demonstrating the different CD4⁺ T cells and CD25⁺CD4⁺ T cells responses between hamster and mouse models when infected with live *Leptospira*. Although there were a similar number of CD4⁺ T cells and CD25⁺CD4⁺ T cells in the primary response, the IFN- γ and IL-4 producing cells were different especially when re-stimulated with LAg or LipL32 antigens. The significantly higher levels of the IFN- γ ⁺ and IL-4⁺CD4⁺ T cells in hamsters might make them to be more susceptible of such infections. The mechanisms of this phenomenon remain to be elucidated when reagents for hamsters are more available.

Acknowledgements

We would like to acknowledge financial supports from Faculty of Medicine and Melioidosis Research Center, Khon Kaen University. Yaowarin Nakornpakdee was supported by The Development and Promotion of Science and Technology Talents Project (DPST). We would like to acknowledge Emeritus Professor James A. Will, University of Wisconsin-Madison, under Publication Clinic, KKU, Thailand for editing this manuscript and Assistant Professor Dr. Onrapak Reamtong, Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University for analyzing rLipL32 peptides by mass spectrometry.

Conflict of Interest

none

Source of Funding with Grant Number

Faculty of Medicine, Melioidosis Research Center, Khon Kaen University, Higher Education Research Promotion and National Research University Project of Thailand, CHE, through the Health Cluster (SheP-GMS) and The Development and Promotion of Science and Technology Talents Project (DPST).

Author Contributions

- Yaowarin Nakornpakdee did almost all experiments.
- Rasana W Sermswan designed the leptospiral experiments.
- Santi Maneewatcharangsri designed, cloned, and expressed rLipL32.
- Surasakdi Wongratanacheewin designed all experiments except leptospiral work, wrote grants, and wrote and edited the manuscript.

References

1. da Silva JB, Ramos TM, de Franco M, Paiva D, Ho PL, Martins EA, et al. Chemokines expression during *Leptospira interrogans* serovar Copenhageni infection in resistant BALB/c and susceptible C3H/HeJ mice. *Microb Pathog*. 2009;47(2):87-93.
2. Adler B. Pathogenesis of leptospirosis: cellular and molecular aspects. *Vet Microbiol*. 2014;172:353-8.
3. Adler J, Jarvis K, Mitten M, Shipkowitz NL, Gupta P, Clement J. Clarithromycin therapy of experimental *Treponema pallidum* infections in hamsters. *Antimicrob Agents Chemother*. 1993;37(4):864-7.
4. Rouault E, Lecoeur H, Meriem AB, Minoprio P, Goyard S, Lang T. Imaging visceral leishmaniasis in real time with golden hamster model: Monitoring the parasite burden and hamster transcripts to further characterize the immunological responses of the host. *Parasitol Int*. 2017;66(1):933-9.
5. Kaewraemruaen C, Sermswan RW, Wongratanacheewin S. Induction of regulatory T cells by *Opisthorchis viverrini*. *Parasite Immunol*. 2016;38(11):688-97.
6. Gomes-Solecki M, Santecchia I, Werts C. Animal Models of Leptospirosis: Of Mice and Hamsters. *Front Immunol*. 2017;8:58.
7. Domingos RH, Pavanel EB, Nakajima E, Schons-Fonseca L, Da Costa RMA, De Franco M, et al. Resistance of mice to *Leptospira* infection and correlation with chemokine response. *Immunobiology*. 2017;222(11):1004-13.
8. Faisal SM, McDonough SP, Chang Y. *Leptospira*: Invasion, Pathogenesis and Persistence. In: Embers ME, editor. *The Pathogenic Spirochetes: Strategies for Evasion of Host Immunity and Persistence*. New York: Springer; 2012. p. 143-72.
9. Volz MS, Moos V, Allers K, Luge E, Mayer-Scholl A, Nockler K, et al. Specific CD4⁺ T-Cell Reactivity and Cytokine Release in Different Clinical Presentations of Leptospirosis. *Clin Vaccine Immunol*. 2015;22(12):1276-84.
10. Tansuphasiri U, Deepradit S, Phulsuksombati D, Tangkanakul W. Two simple immunoassays using endemic leptospiral antigens for serodiagnosis of human leptospirosis. *Southeast Asian J Trop Med Public Health*. 2005;36:302-11.
11. Maneewatch S, Sakolvaree Y, Saengjaruk P, Srimanote P, Tapchaisri P, Tongtawe P, et al. Monoclonal antibodies to LipL32 protect against heterologous *Leptospira* spp. challenge. *Hybridoma (Larchmt)*. 2008;27:453-65.
12. Maneewatch S, Adisakwattana P, Chaisri U, Saengjaruk P, Srimanote P, Thanongsaksrikul J, et al. Therapeutic epitopes of *Leptospira* LipL32 protein and their characteristics. *Protein Eng Des Sel*. 2014;27:135-44.
13. Fraga TR, Barbosa AS, Isaac L. Leptospirosis: aspects of innate immunity, immunopathogenesis and immune evasion from the complement system. *Scand J Immunol*. 2011;73(5):408-19.
14. Dondji B, Bungiro RD, Harrison LM, Vermeire JJ, Bifulco C, McMahon-Pratt D, et al. Role for nitric oxide in hookworm-associated immune suppression. *Infect Immun*. 2008;76(6):2560-7.
15. Hammerbeck CD, Hooper JW. T cells are not required for pathogenesis in the Syrian hamster model of hantavirus pulmonary syndrome. *J Virol*. 2011;85(19):9929-44.
16. Adler B. History of leptospirosis and leptospira. *Curr Top Microbiol Immunol*. 2015;387:1-9.
17. Matsui M, Rouleau V, Bruyere-Ostells L, Goarant C. Gene expression profiles of immune mediators and histopathological findings in animal models of leptospirosis: comparison between susceptible hamsters and resistant mice. *Infect Immun*. 2011;79(11):4480-92.
18. Srikram A, Wongratanacheewin S, Puapairoj A, Wuthiekanun V, Sermswan RW. Analyses of vaccination protocols for *Leptospira interrogans* serovar Autumnalis in hamsters. *Am J Trop Med Hyg*. 2008;79:779-86.
19. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, et al. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect Immun*. 2000;68(4):2276-85.
20. Matsui M, Soupe ME, Becam J, Goarant C. Differential *in vivo* gene expression of major *Leptospira* proteins in resistant or susceptible animal models. *Appl Environ Microbiol*. 2012;78(17):6372-6.