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Research article

Title: High level of IL-10 expression in the blood of animal models possibly relates to resistance against leptospirosis

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Abstract

Leptospirosis is a severe zoonosis which immunopathogenesis is poorly understood. We evaluated correlation between acute form of the disease and the ratio of the anti-inflammatory cytokine IL-10 to the pro-inflammatory TNF- α and IL-1 β expression during the early phase of infection comparing resistant mice and susceptible hamsters infected with two different species of virulent *Leptospira*. The IL-10/TNF- α and IL-10/IL-1 β expression ratios were higher in mouse compared to hamster independently of the *Leptospira* strain, suggesting a preponderant role of the host response and notably these cytokines in the clinical expression and survival to leptospirosis. Using an IL-10 neutralization strategy in *Leptospira*-infected mouse model, we also showed evidence of a possible role of this cytokine on host susceptibility, bacterial clearance and on regulation of cytokine gene expression.

Keywords: leptospirosis; *in vivo*; cytokine; IL-10; IL-1 β ; TNF- α

Highlights

- Higher IL-10/TNF- α and IL-10/IL-1 β expression ratios in mouse compared to hamster.
- Differential expression independently of the *Leptospira* strain.
- Neutralisation of IL-10 led to increased susceptibility in *Leptospira*-infected mouse.
- IL-10 has a role in regulation of IL-1 β during leptospirosis.
- IL-10 neutralization did not interact with TNF- α expression level.

1. Introduction

Mainly reported in tropical and developing countries, leptospirosis is a zoonosis caused by pathogenic *Leptospira*, and recent estimations revised the annual number of human cases over one million infection cases with almost 60,000 deaths [1]. Contamination occurs after contact with bacteria shed in the urine of reservoir animals, mostly rodents [2] and human leptospirosis is characterized by various clinical symptoms that can degenerate to severe forms with haemorrhages and multiple organ failure [3].

Severe human leptospirosis presents evidence of a “cytokine storm” process characterized by increased levels of pro- (TNF- α , IL-6) and anti- (IL-10) inflammatory cytokines in serum of patients [3] and several inflammatory mediators were investigated as biomarkers of severity in human cases. Interestingly, high ratio of IL-10 to TNF- α circulating level was correlated to severity of human leptospirosis [4, 5]. However, contradictory results were also reported showing that higher ratio of IL-10 to TNF- α serum levels was positively correlated with fatal outcome [6, 7]. Interestingly, the last study of Chirathaworn and coll. did not show significant difference of IL-10/TNF- α ratio in leptospirosis patients with or without organ involvement [8]. Previously, we demonstrated that gene expression levels of TNF- α and IL-10 in blood were significantly higher and related to lethality in a susceptible infection model using hamsters [9]. We also studied the cytokine gene expression in the blood of resistant mice, and, while pro-inflammatory cytokines were restored to basal levels during the early days postinfection, IL-10 was rapidly overexpressed [10].

In this study, we performed further experiments *in vivo* to clarify the regulation of cytokines IL-10, IL-1 β and TNF- α , in animal models of experimental leptospirosis, also investigating tissue lesions and bacterial burden. Mice were used as asymptomatic models, either regarded as resistant when infected with *L. interrogans* serovar Icterohaemorrhagiae strain Verdun (LiVV) or as a typical reservoir model when inoculated with a *L. borgpetersenii*

serogroup Ballum. Hamsters were used to reproduce severe leptospirosis when infected with either *Leptospira* strain. As infecting serovars can affect the severity of human leptospirosis [11], two different species of virulent leptospires were used for infection. Moreover, to better assess the acute phase of infection in the susceptible model, gene expression was investigated between 3 and 4 days (D) postinfection in the blood of animals, also corresponding to high leptospiremia in susceptible hamsters. The ratios of IL-10/TNF- α and IL-10/IL-1 β were investigated according to animal survival or fatal outcome, histological changes in organs and bacterial burden in blood. We then investigated the possible involvement of the immunosuppressive IL-10 in leptospirosis pathogenesis using an IL-10 neutralization strategy in *Leptospira*-infected mouse model to monitor its possible effect on host susceptibility, bacterial clearance and on regulation of cytokine gene expression.

2. Material and Methods

2.1. Animals and bacterial strains

Outbred OF1 mice (*Mus musculus*) and golden Syrian hamsters (*Mesocricetus auratus*), initially obtained from Charles River Laboratories, were bred in animal facility in Institut Pasteur in New Caledonia. Virulent *L. interrogans* serovar Icterohaemorrhagiae strain Verdun (LiVV) or *L. borgpetersenii* serogroup Ballum isolate B3-13S (B3-13S) were cultured in EMJH medium as previously described [12]. Virulence of leptospires was maintained and verified by monthly passages in 6- to 8-week-old golden Syrian hamsters after intraperitoneal injection before re-isolation from blood. Animal manipulations were conducted according to the guidelines of the Animal Care and Use Committees of the Institut Pasteur and followed European Recommendation 2007/526/EC.

2.2. Comparative experimental infection

For comparative study between *Leptospira* strains, 6- to 8-week-old healthy animals ($n \geq 5$ individuals per condition) were infected and experiments were carried out as previously described [12]. Briefly, mice and hamsters were injected intraperitoneally with 2×10^8 leptospires in EMJH medium. Non-infected animals served as controls. After euthanasia with chloroform or by inhalation of CO_2 , whole blood was collected between D3 and D4 postinfection by cardiac puncture and conserved in PAXgene blood RNA tubes (PreAnalytiX, Qiagen) for molecular biology. Sera of LiVV-infected mice were collected at D3 and D7 after infection for protein quantitation. Organs were fixed in 10% neutral buffered formalin for histology.

2.3. IL-10 neutralization challenge

For evaluation of IL-10 neutralization in murine models, 5-week old OF1 mice were injected with 200 μg of anti-IL-10 antibodies [13] in 200 μL of PBS (LEAF Purified anti-mouse IL-10, rat IgG1, κ from clone JES5-2A5, or rat IgG2b, κ from clone JES5-16E3, BioLegend, Australia) 3 hours prior to infection with 2×10^8 leptospires of the strain LiVV in EMJH medium ($\alpha\text{IL-10+LiVV}$ group). Infected animals without antibodies treatment were injected with 200 μL of PBS (PBS+LiVV group) to reproduce double injection. Mice injected with anti-IL-10 and EMJH medium were also included ($\alpha\text{IL-10+EMJH}$ group). Animals without injection served as control. One hamster was also infected with the same dose of bacteria to confirm virulence and lethality of the strain during experiment. Body weight of mice was monitored prior to injections and until D7 postinfection. Animals were observed for survival and for any signs of clinical illness (loss of appetite or reactivity, prostration, ruffled fur, external hemorrhage). Blood samples (50 μL) were non-lethally collected by caudal vein puncture at D3 postinfection and processed as detailed above using the PAXgene blood RNA system (PreAnalytiX, Qiagen). Animals were humanely killed at D7 postinfection and kidneys were rapidly collected for

molecular bioassays (within 5 minutes of euthanasia) for extraction of total DNA. Briefly, 25 mg samples were placed into MagNA Lyser Green Beads tubes (Roche Applied Sciences) containing 360 μ L lysis buffer (QIAamp DNA Mini kit, Qiagen).

2.4. Histology

Paraffin-embedded sections were stained with hematoxylin-erythrosin (HE). Morphological changes in liver, lungs and kidneys were analyzed and lesions were graded blindly according to previously reported criteria [10]. Average scores were totalized ranging from normal (0) to severe (20) for each individual.

2.5. Purification of DNA

Total DNA was extracted using QIAamp DNA Mini kit (Qiagen) from renal tissues. Tissue samples in MagNA Lyser Green Beads tubes were first disrupted and homogenized using the MagNA Lyser Instrument (Roche Applied Science) during 50 s at 7,000 r/min. Lysates were incubated at 56°C for 1 h with 190 μ L of PBS and 50 μ L of proteinase K. After washing steps, the eluted DNA was quantified by spectrophotometry (NanoDrop 2000, ThermoFisher).

2.6. Reverse Transcription

For gene expression, total RNA was purified using the PAXgene blood RNA system before treatment with DNase (Turbo DNA-free kit; Ambion, Applied Biosystems), and cDNA synthesized using Transcriptor First Strand cDNA synthesis kit (Roche Applied Science) as previously described [10].

2.7. Quantitative PCR

Primers and probes were purchased from Eurogentec (Seraing, Table 1). Renal DNA was amplified using the LightCycler FastStart DNA Master SYBR Green I and targeted the *lfb1* gene [14] on a LightCycler 2.0 (Roche Applied Science). Alternatively, bacterial load was assessed using the LightCycler 480 Probe Master targeting *lipL32* gene on a LightCycler 480 II instrument (software v.1.5.0; Roche Applied Science) [15]. Genomic DNA from corresponding leptospire was used as a positive control. Quantitative PCR for IL-10, IL-1 β , TNF- α , *Leptospira* 16S rRNA and the reference genes glyceraldehyde-3-phospho- deshydrogenase (GAPDH) and β -actin were conducted on the LightCycler 480 II using the cDNA previously generated and the LightCycler 480 SYBR Green I Master kit as previously described [10]. Normalization of expression was processed using qbase PLUS software (Biogazelle) by extracting the expression levels of the reference genes. The normalized expression ratio of *Leptospira* 16S rRNA was calculated for evaluation of bacteria level. For cytokine gene expression, the relative normalized expression ratio was then calculated as the ratio of the expression level in infected animals to the expression level in control animals.

2.8. Quantification of cytokines (xMAP technology)

Interleukin-10, IL-1 β and TNF- α levels in serum of mouse were quantified using a multiplex magnetic bead-based immunoassay (MyCytoMag 70k-10.Mouse kit, Merck-Millipore), according to manufacturer's instructions. The specific bead fluorescence was measured by a MAGPIX® instrument (Luminex, Austin, USA). The sample concentrations were calculated using a 10-parameter logistic standard curve using the MILLIPLEX® Analyst 5.1 software (Merck Millipore, Billerica, USA).

2.9. Statistical analyses

Statistical studies were performed using GraphPad Prism v4 (GraphPad Software Inc.). Mann-Whitney test was used for analysis of significant differences in histopathological score, bacterial load and cytokine gene expression between animals. A Two-way ANOVA test was used to compare difference in body weight variation depending on treatment between mice during IL-10 neutralization challenge.

3. Results

3.1. Tissue lesions and leptospiremia in *Leptospira*-infected animals

Animals were infected with LiVV or B3-13S strain and fatal outcome was observed in hamsters within four to six days after inoculation as previously shown [9, 12]. Severe alterations were observed in B3-13S-infected hamster organs with diffuse alveolar haemorrhages, oedema and pulmonary congestion with inflammatory infiltrates in lungs (Figure 1A), interstitial nephritis, glomerular congestion and tubular haemorrhages in kidneys (Figure 1B), and hepatocytic necrosis, sinusoidal congestion (Figure 1C), portal inflammation and hyperplasia of Kupffer cells in liver. Similar observations were reported in LiVV-infected hamsters (data not shown). In contrast, no or mild morphological changes were observed in *Leptospira*-infected mice (data not shown).

Histopathological scores (Figure 1D) showed significantly more severe lesions in hamsters compared to mice infected with LiVV (mean score $\pm$ SE at 5.33 ± 0.95 and 13.17 ± 1.33 in mice and in hamsters, respectively) or with B3-13S (0.15 ± 0.15 and 7.0 ± 3.41 in mice and in hamsters, respectively). Bacterial burden was investigated in the blood of animals during the onset of the symptoms between three and four days postinfection (Figure 1E) and, as expected, bacterial load was significantly higher in hamster blood (mean $\pm$ SE at 17.06 ± 6.31 and 0.57 ± 0.18 for infection with LiVV and B3-13S, respectively) compared to mice (3.41 ± 1.20 and $<1E-4$ for infection with LiVV and B3-13S, respectively) regardless of the infecting strain.

3.2. Cytokine gene expression in blood of animals depending on infecting strains

Gene expression level of cytokines was quantified in the blood of animals and IL-10 gene expression (Figure 2A) was significantly higher at D3-D4 postinfection in the blood of infected mice compared to hamsters (mean ratios of 18.95 ± 7.15 compared to 5.07 ± 1.51 respectively for LiVV infection or 80.19 ± 21.67 compared to 17.34 ± 3.33 respectively for B3-13S infection). Significant overproduction of IL-10 was confirmed in sera of mice infected with LiVV 3 days after infection (265.0 ± 89.72 pg/mL) compared to control level (7.23 ± 0.48 pg/mL; Figure 2D). Blood IL-10 returned to basal level at D7 postinfection (8.12 ± 1.38 pg/mL). Unfortunately, commercial kits are not available for quantification of cytokines in the blood from Syrian hamster.

Gene expression of TNF- α was also significantly up-regulated at D3-D4 in both mice and hamsters (Figure 3A) infected with LiVV (means $\pm$ SE of expression ratio at 2.46 ± 0.84 and 3.31 ± 0.40 in mice and hamsters, respectively) or with B3-13S strain (9.26 ± 2.59 and 15.07 ± 2.70 in mice and hamsters, respectively). Interestingly, no difference was observed between animals irrespective of strains. All IL-1 β expression levels were up-regulated and significantly different compared to control in animals infected with LiVV (ratio at 3.75 ± 1.8 in hamsters) or with B3-13S strain (10.06 ± 4.66 in mice and 9.95 ± 5.17 in hamsters, respectively), except for LiVV-infected mice (Figure 3B). However, contrasting with TNF- α , significant difference was observed in IL-1 β expression between mice and hamsters during LiVV infection, with higher expression in hamsters.

Both IL-10/TNF- α and IL-10/IL-1 β ratios of gene expression values in blood (Figure 3C-D) were significantly higher in mice (25.13 ± 11.90 and 11.91 ± 3.44 with LiVV and B3-13S, respectively for IL-10/TNF- α , and 13.99 ± 7.49 and 7.99 ± 4.21 with LiVV and B3-13S, respectively for IL-10/IL-1 β) than in hamsters (1.44 ± 0.29 and 2.39 ± 0.99 with LiVV and B3-

13S, respectively for IL-10/TNF- α ratio, and 1.31 ± 0.51 and 2.626 ± 1.19 with LiVV and B3-13S, respectively for IL-10/IL-1 β). Interestingly, cytokine gene expression level was significantly different depending on the strain used for infection among mice or hamsters (Figure 3A-B) but no effect of strain was observed when comparing expression ratios IL-10/TNF- α or IL-10/IL-1 β (Figure 3C-D). Cytokine concentrations in sera of mice were also quantified using multiplex technology, and TNF- α and IL-1 β were not different between control and infected mice at D3 postinfection possibly related to low expression level (data not shown).

3.3. Characterization of LiVV infection after IL-10 neutralization in mice

In order to investigate the role of IL-10 in pathogenesis, anti-IL-10 neutralizing antibodies were injected mice prior to their infection. LiVV strain was used and animals were double injected either with PBS or with EMJH to evaluate potential activity of solutions. All mice survived during experimental infection. However, lower reactivity and mobility, and loss of appetite were observed in LiVV-infected mice challenging IL-10 neutralization during the first 2 days postinfection while no clinical signs were observed for the other animal groups. Body weight of animals (Figure 4A) was monitored to assess the effect of IL-10 neutralization on leptospirosis severity and bacterial load. No significant difference was observed for double injected PBS+LiVV or for α IL-10+EMJH mice compared to non-injected control animals, confirming natural resistance of OF1 mouse to LiVV infection. In contrast, α IL-10+LiVV mice showed a significant decrease in body weight (percentage of initial body weight of 94.14 ± 2.80 % and 98.43 ± 3.17 % at D1 and D2 postinfection, respectively) when compared to PBS+LiVV injected animals (99.42 ± 2.78 and 103.3 ± 3.60 at D1 and D2 postinfection, respectively).

Leptospiemia was also measured in blood of infected mice and no difference was observed during IL-10 neutralization challenge at D3 postinfection (Figure 4B). Presence of leptospires

was investigated and bacterial load was also quantified in kidneys of animals at D7 postinfection (Figure 4C). Interestingly, leptospire were detected in 1 / 7 mice in kidneys from LiVV-infected mice pre-injected with anti-IL-10 antibodies while 5 / 7 animals were positive in PBS+LiVV group. Moreover, leptospiral load was lower in α IL-10+LiVV mice (6.27 ± 16.59) compared to PBS+LiVV animals (552.20 ± 760.80) supporting a possible effect of IL-10 neutralization on bacterial dissemination or clearance in mice kidneys.

3.4. Effect of IL-10 neutralization on *Leptospira*-dependent cytokine gene expression in mouse

Regulation of cytokine gene expression was investigated in blood of LiVV-infected mice challenging IL-10 neutralization at D3 postinfection (Figure 5). Up-regulation of IL-10 gene expression was confirmed in infected animals compared to control group (mean expression ratio at 14.98 ± 3.87 in PBS+LiVV group; Figure 5A) without significant effect of anti-IL-10 antibodies treatment on LiVV-dependent gene overexpression (ratio at 10.84 ± 4.72). Similarly, TNF- α was overexpressed in blood of infected mice compared to control but no significant difference was observed between LiVV-infected animals with or without anti-IL-10 antibodies pre-injection (Figure 5B). A different expression pattern was observed for IL-1 β transcript level (Figure 5C) with no significant overexpression of this cytokine in blood from PBS+LiVV group (mean expression ratio at 1.45 ± 0.32) compared to control animals, comforting previous results (Figure 3B). In contrast, IL-1 β gene expression was significantly upregulated in blood from LiVV-infected mice challenging IL-10 neutralization (ratio at 2.12 ± 0.24) compared to control group, and significantly different compared to PBS+LiVV animals.

4. Discussion

Cytokines constitute key inflammatory mediators that orchestrate immune responses to infection [16] and differential regulation of cytokine expression was related to clinical outcomes in human leptospirosis. Expression levels of cytokines including IL-10, IL-1 β and TNF- α were previously evaluated as potential markers of leptospirosis severity in human, and studies showed contrasting results regarding ratio of IL-10 to TNF- α correlated with disease severity [4-8]. This might be explained by difference in infecting strains. Indeed, using a hamster model, Fujita and collaborators showed differential regulation of cytokine gene expression depending on the infecting serovars [17]. Thus, we analysed regulation of IL-10, IL-1 β and TNF- α in blood from susceptible hamsters and resistant mice infected with two different species of leptospires to investigate effect of strains on cytokine expression, and relation between animal resistance and cytokine regulation. Except for IL-1 β in LiVV-infected mice, gene expression of cytokines was up-regulated in blood of animals at D3-4 postinfection compared to control level. Interestingly, IL-10, IL-1 β and TNF- α expression levels but not the IL-10/TNF- α nor IL-10/IL-1 β expression ratios were differentially regulated within the same animal model depending on inoculated leptospires. Infecting strains might differentially affect each cytokine gene expression while IL-10/TNF- α and IL-10/IL-1 β ratios appear independent of leptospire serovars, suggesting these ratios as possible indicators of leptospirosis severity.

Less tissue lesions and lower leptospiremia with higher cytokine expression levels were observed in B3-13S- compared to LiVV-infected animals and it is noticeable that B3-13S leptospires belong to the serovar Ballum which natural reservoirs are mice [11]. Thus, infection of mice with their host-coadapted serovar also promotes inflammatory responses with upregulation of cytokine levels during the early phase of infection. This also suggests virulence of strain as a preponderant factor of cytokine regulation possibly affecting observations and prognosis in human cases as previously shown [18]. Conversely, cytokine regulation could be regarded as a major actor of leptospirosis pathophysiology. Pro-inflammatory TNF- α and anti-

inflammatory IL-10 are amongst the main biological mediators exerting adverse effects during bacterial infection and both cytokines are induced during experimental leptospirosis as previously shown [10]. However, no difference was observed in TNF- α expression level between hamster and mouse when infected with the same strain. This correlates with observations in leptospirosis patients of whom TNF- α although induced in blood, was not different depending on organ involvement or severity [5, 8]. Oppositely, IL-10 level was higher in mouse blood compared to hamster blood irrespective of the strain. This suggests a preponderant role of this anti-inflammatory cytokine in the resistance of mice to leptospirosis. That is reflected by a higher IL-10/TNF- α and IL-10/IL-1 β ratios in resistant mouse compared to susceptible hamster. As a limitation, evaluation of gene expression at the transcript level might not correlate with protein regulation due to possible post-transcriptional regulation mechanisms. Thus, we performed quantification of cytokine concentration in blood of LiVV-infected mice and confirmed the increase of IL-10 levels in this resistant mouse model. Assessment of cytokine concentration in hamster blood would also be of interest but specific antibodies are hardly available for hamsters.

Interleukin-10 plays a major role in the resolution of inflammation during sepsis and infection avoiding appearance of deleterious lesions but is also involved in persistence of pathogens by interfering with innate and adaptive immunity. Indeed, this molecule acts on several types of leucocytes including antigen-presenting cells (APCs), by downregulating expression of co-stimulatory markers and production of proinflammatory cytokines [19]. It also directly inhibits T-cell response and proliferation while down-regulating expression of Th1 type cytokines, IL-2 and interferon- γ (IFN- γ) [20]. Interestingly, in an IL-10 deficient murine model of Lyme disease, sustained IFN- γ production was observed resulting in development of disease related arthritis [21]. In parallel, IL-10 also has the potential to stimulate B cells contributing to immunoglobulin class switching in these leucocytes [22]. Considering that IL-

10 is a multipotent anti-inflammatory cytokine with different facets closely associated with susceptibility or resistance to infections and microbial persistence, we investigated the role of IL-10 in leptospirosis pathogenesis using a pre-neutralization strategy by injecting anti-IL-10 antibodies in LiVV-infected mouse. Although not lethal, IL-10 pre-neutralization in *Leptospira*-infected mice led to the development of a disease in this usually resistant model with observations of clinical signs and weight loss during onset of the disease.

To better assess the role of IL-10 in the regulation of immune response occurring during leptospirosis, effect of IL-10 previous neutralization was also evaluated on the regulation on *Leptospira*-dependent cytokine gene expression. Surprisingly, no effect of IL-10 neutralization was observed on LiVV-induced TNF- α transcript level. Experimental infection of C57BL/6 mice deficient for TNF- α receptor (TNFR) injected with pathogenic *L. interrogans* serovar Copenhageni exhibited more severe renal lesions suggesting this cytokine as an important mediators in the early control of infection [23]. Similarly, no difference was observed in TNF- α expression level between resistant mouse and susceptible hamster during *Leptospira* infection (Figure 3A). Contrasting with TNF- α results, IL-10 neutralization seemed to potentiate IL-1 β overexpression in LiVV-infected mice as observed for LiVV-infected susceptible hamsters. Previous studies highlighted importance of pro-inflammatory cytokine IL-1 β through overstimulation of the inflammasome system that could contribute to tissue damages [24]. Leptospiral glycolipoprotein was also shown to activate the inflammasome system through modulation of potassium transport and, together with *Leptospira* lipopolysaccharide, contribute to the IL-1 β overexpression and production induced by *L. interrogans* infection in susceptible murine models [25]. Similarly, high IL-1 β expression was associated with fatal outcome from pulmonary haemorrhages in canine leptospirosis [26]. Considering that IL-1 β overexpression might be related to susceptibility and organ failures in *Leptospira*-infected host, the immuno-

protective role of IL-10 might be effective by regulating inflammatory response during leptospirosis counterbalancing deleterious activities of pro-inflammatory IL-1 β .

Effect of IL-10 neutralization was also evaluated on bacterial clearance and leptospiral burden in blood and kidneys from LiVV-infected mice. Although no impact was observed in leptospiremia, results showed evidences of IL-10 role in bacterial clearance in renal tissues. Indeed, neutralization of IL-10 in *Leptospira*-infected mice led to a decrease in bacterial burden in kidneys at D7 postinfection. Interestingly, contribution of IL-10 in the attenuation of the inflammatory lesions during Lyme disease was shown but, interestingly, inhibition of endogenous IL-10 also enhanced bacterial elimination [27]. Thus, IL-10 might be a major component in the maintenance of *Leptospira* in the kidneys of reservoir mouse in its natural environment.

This study suggests that the regulation of cytokine gene expression, especially the anti-inflammatory IL-10, could be of importance in the leptospirosis pathogenesis. Indeed, lower levels of IL-10 were shown in the blood of hamsters lethally infected regardless of the *Leptospira* species used for inoculation. Moreover, inhibition of endogenous IL-10 in *Leptospira*-infected mouse presented evidences of the dual role of this cytokine by regulating immune response occurring during leptospirosis but exerting inhibitory effect on effective bacterial clearance.

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Figures and captions

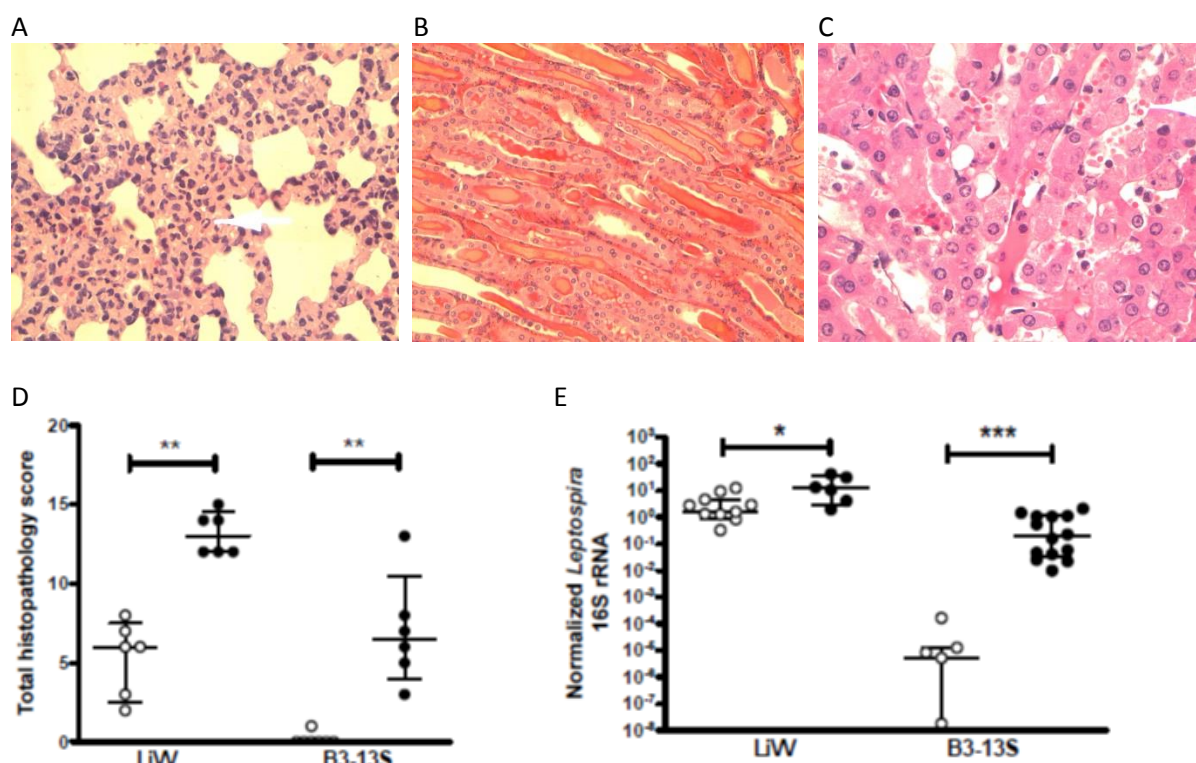


Figure 1. Characterization of leptospirosis infection in resistant and susceptible animal models injected with virulent *Leptospira* species. Microscopic observations of tissues lesions in B3-13S infected hamster were observed with intense congestion in all organs coming along with infiltration of polymorphonuclear cells in lungs (A, open arrow), diffuse hemorrhages in renal tubules (B), and sinusoidal dilatation in liver (C, open arrow) at D4 postinfection (HE, x400). Results present also histopathological score in organs (D), and quantification of *Leptospira* 16S rRNA in blood (E) from mice (open circles) and hamsters (filled circles) at D3-4 after infection with virulent *L. interrogans* serovar Icterohaemorrhagiae strain Verdun (LiVV) or *L. borgpetersenii* serovar Ballum isolate B3-13S (B3-13S) as described in Methods. Shown is the median (horizontal bars) $\pm$ interquartile ranges and individual data (dots). Significant difference between animals was evaluated using a non-parametric Mann-Whitney test. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0005$, ns: not significant.

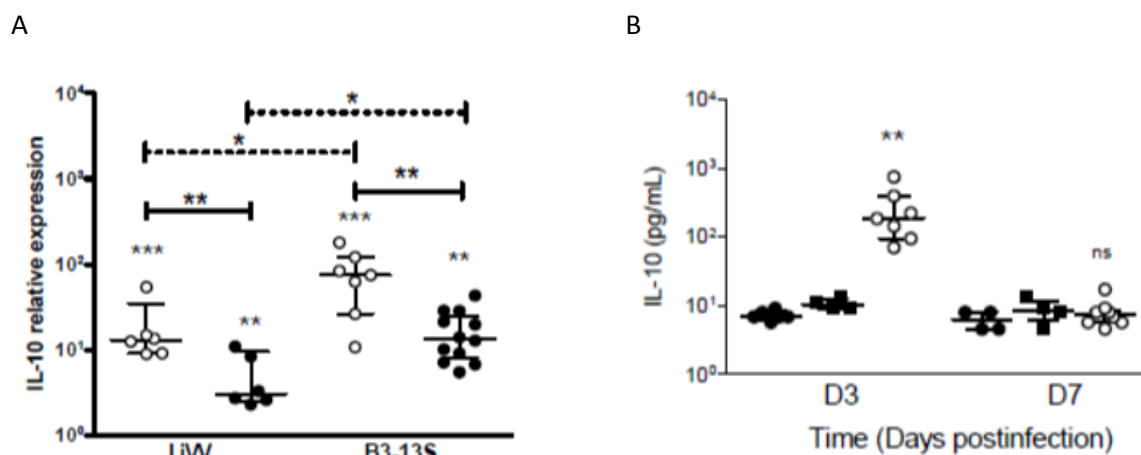


Figure 2. Differential overexpression of IL-10 in blood of animals injected with virulent *Leptospira* species. Results present relative gene expression of IL-10 (A) in blood from mice (open circles) and hamsters (filled circles) at D3-4 after infection with virulent *L. interrogans* serovar Icterohaemorrhagiae strain Verdun (LiV) or *L. borgpetersenii* serovar Ballum isolate B3-13S (B3-13S). Protein level of IL-10 (B) was quantified in blood from mice infected with LiV (open circles) compared to uninfected (filled circles) or EMJH-injected (filled squares) control animals. Shown is the median (horizontal bars) $\pm$ interquartile range and individual data (dots). Significant difference was evaluated compared to basal level in uninfected animals (above asterisks), between animal models for each strain (solid lines) or between strains for each animal model (dotted lines) using a non-parametric Mann-Whitney test. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0005$, ns: not significant.

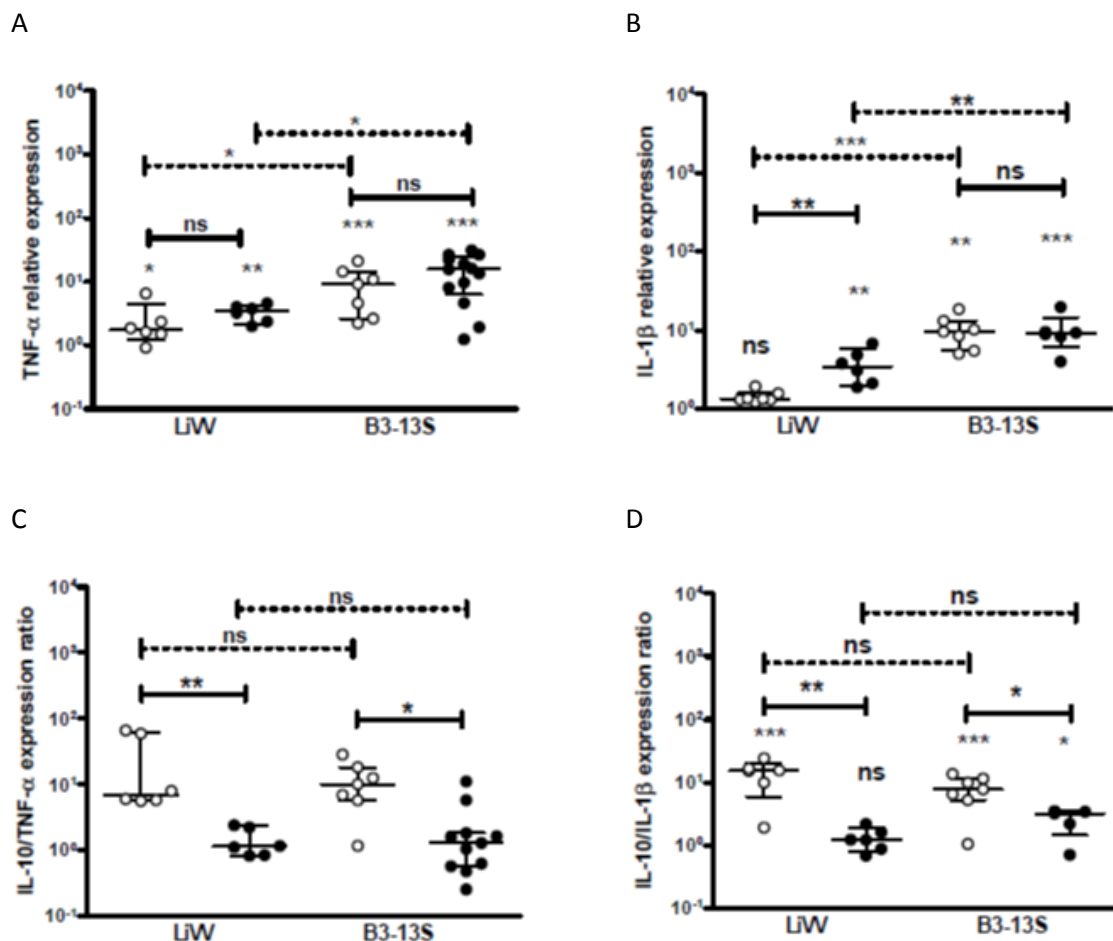


Figure 3. Regulation of TNF- α and IL-1 β gene expression in blood of animals injected with virulent *Leptospira* species. Results present relative gene expression of TNF- α (A) and IL-1 β (B), and IL-10 to TNF- α (C) and IL-10 to IL-1 β (D) expression ratio in blood from mice (open circles) and hamsters (filled circles) at D3-4 after infection with virulent *L. interrogans* serovar Icterohaemorrhagiae strain Verdun (LiVv) or *L. borgpetersenii* serovar Ballum isolate B3-13S (B3-13S). Shown is the median (horizontal bars) $\pm$ interquartile range and individual data (dots). Significant difference was evaluated compared to basal level in uninfected animals (above asterisks), between animal models for each strain (solid lines) or between strains for each animal model (dotted lines) using a non-parametric Mann-Whitney test. * P <0.05, ** P <0.001, *** P <0.0005, ns: not significant.

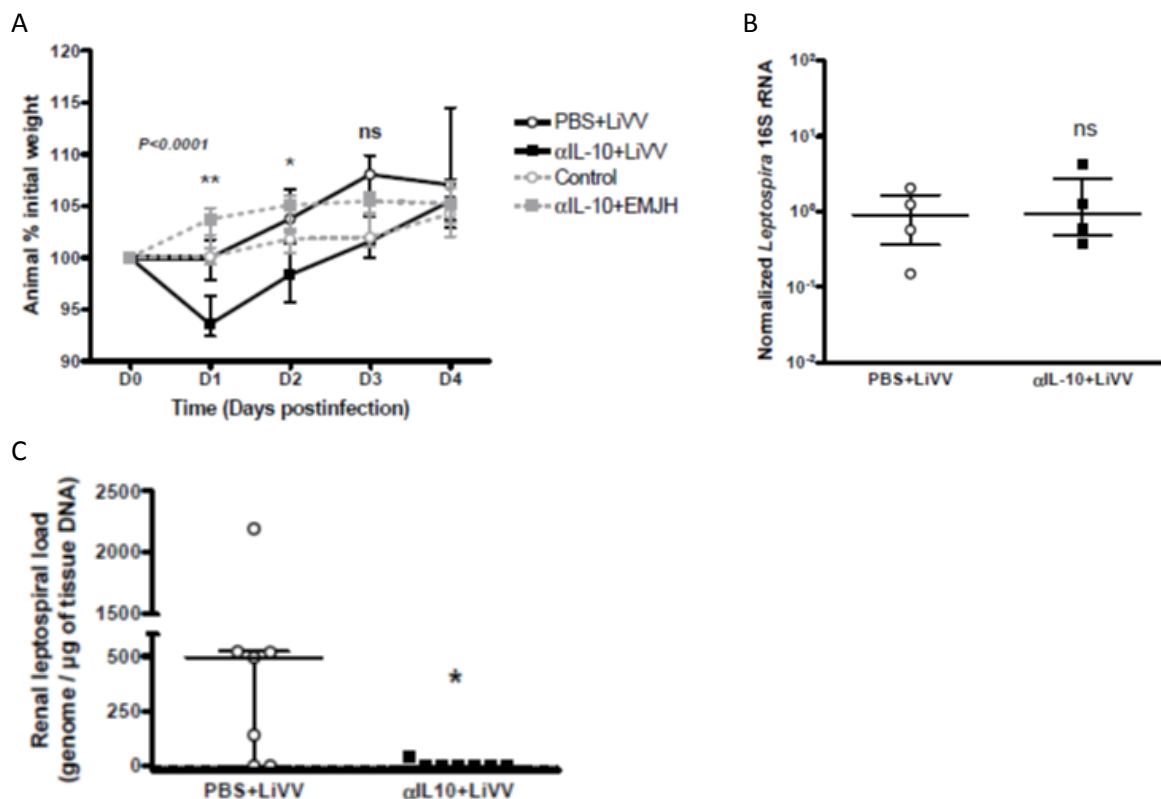


Figure 4. Characterization of leptospirosis infection in mouse challenging IL-10 neutralization. Body weight (A) was monitored in mice ($n = 7$ or 8 individuals) until 4 days after infection with virulent *L. interrogans* serovar Icterohaemorrhagiae strain Verdun (LiVV) and pre-injected with 200 μ L of PBS (PBS+LiVV) or 200 μ g / 200 μ L of anti-IL-10 antibody (α IL-10+LiVV) as described in Methods. Growth curves of control mice without injection (Control) or double injected with anti-IL-10 antibody and EMJH medium are also presented (α IL-10+EMJH). Values represent mean $\pm$ SD. Significant difference between animals was evaluated using a 2-WAY ANOVA test to compare difference between PBS+LiVV and α IL-10+LiVV for all the time challenge ($P < 0.0001$). Significant difference between treatments at each time point was also evaluated using a non-parametric Mann-Whitney test. $*P < 0.05$, $**P < 0.001$, ns: not significant. Quantification of *Leptospira* 16S rRNA in blood at D3 postinfection (B) and bacterial load in kidneys at D7 postinfection (C) were measured in LiVV-infected mice pre-injected with 200 μ L of PBS (PBS+LiVV) or with 200 ng / 200 μ L of anti-IL-10 antibody (α IL-10+LiVV). Shown is the median (horizontal bars) $\pm$ interquartile ranges and individual data (dots). Significant difference between conditions was evaluated using a non-parametric Mann-Whitney test. $*P < 0.05$, ns: not significant.

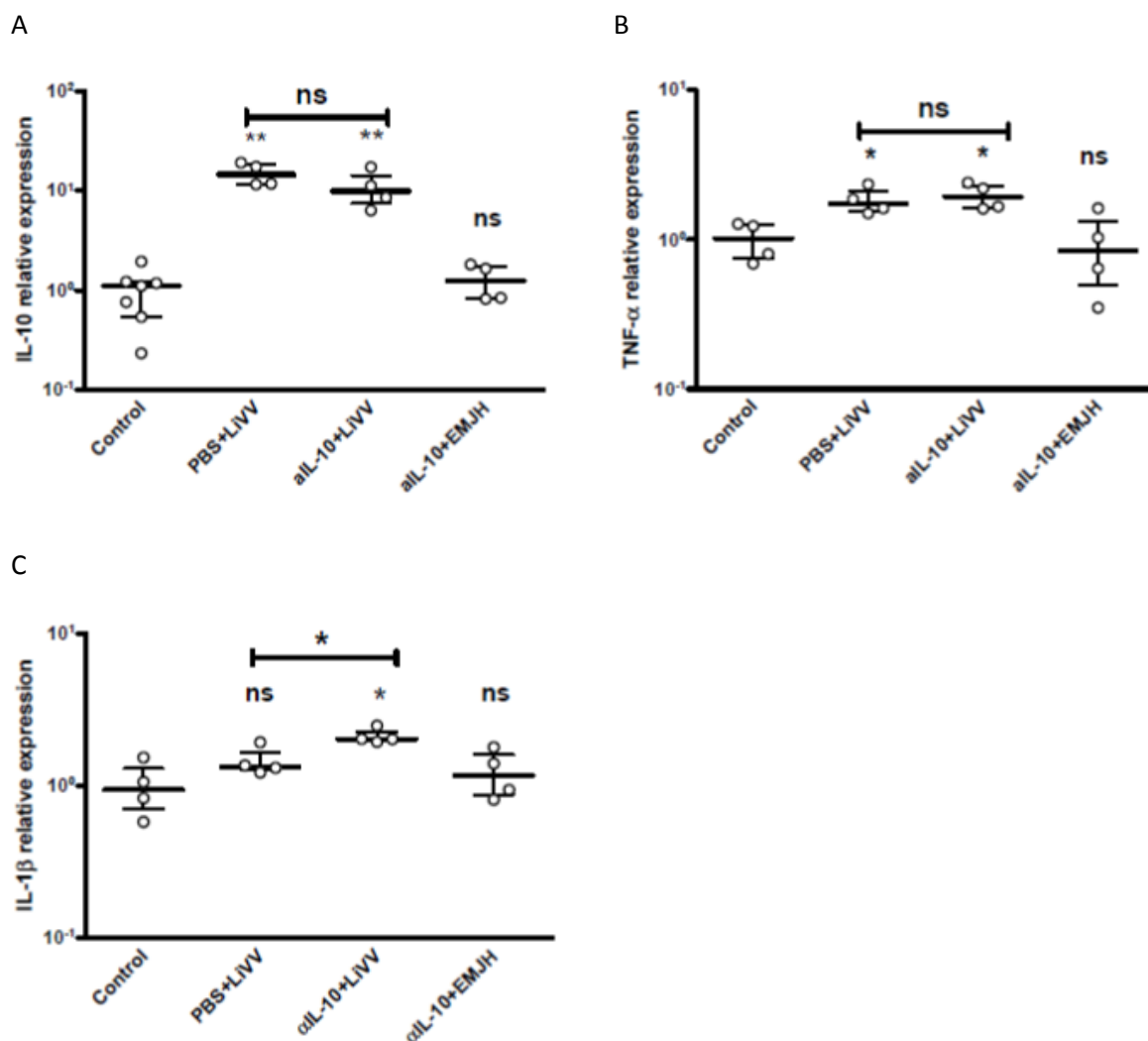


Figure 5. Regulation of cytokine gene expression in blood of LiVV-infected mice challenging IL-10 neutralization. Results present relative gene expression of IL-10 (A), TNF-α (B) and IL-1β (C) in blood from LiVV-infected mice after pre-injection with 200 μL of PBS (PBS+LiVV) or 200 μg / 200 μL of anti-IL-10 antibody (αIL-10+LiVV) at D3-4 postinfection. Blood expression of cytokines in control mice without injection (Control) or double-injected with antibody and EMJH medium (αIL-10+EMJH) were shown. Shown is the median (horizontal bars) ± interquartile range and individual data (dots). Significant difference was evaluated compared to basal level in uninfected animals (above asterisks) or between treatments (solid lines) using a non-parametric Mann-Whitney test. **P*<0.05, ***P*<0.001, ns: not significant.

Table 1. Details and sequence of primers used for qPCR assays

Gene	GenBank ^a	Sequence (5'-3') ^b	Size ^c	Tm ^c	Efficiency ^d
Hamster β -actin	AF046210	(F) TCTACAACGAGCTGCG (R) CAATTTCCCTCTCGGC	357	88.33	1.802±0.04
Murine β -actin	NM_007393	(F) AAGAGAAGCTGTGCTATGTT (R) GTTGGCATAGAGGTCTTTACG	251	86.25	1.660±0.09
Hamster GAPDH	DQ403055	(F) CCGAGTATGTTGTGGAGTCTA (R) GCTGACAATCTTGAGGGA	170	85.67	1.938±0.04
Murine GAPDH	NM_008084	(F) TCATCCAGAGCTGAACG (R) GGGAGTTGCTGTTGAAGTC	213	86.39	1.853±0.02
Hamster IL-10	AF046210	(F) TGGACAACATACTACTCACTG (R) GATGTCAAATTCATTCATGGC	308	85.50	1.871±0.05
Murine IL-10	NM_010548	(F) ATTCCTGGGTGAGAAG (R) CTCTGTCTAGGTCCTGG	259	83.45	1.891±0.04
Hamster IL-1 β	AB028497.1	(F) ATCTTCTGTGACTCCTGG (R) GGTTTATGTTCTGTCCGT	156	85.29	1.852±0.04
Murine IL-1 β	NM_008361	(F) GTGTGGATCCCAAGCAATAC (R) GTTGTTCAGGAAGACAG	161	83.35	1.660±0.09
Hamster TNF- α	AF046215	(F) AACGGCATGTCTCTCAA (R) AGTCGGTCACCTTTCT	278	88.05	1.849±0.03
Murine TNF- α	NM_013693	(F) CAACGGCATGGATCTCA (R) GGAATCCGCAAGTCT	325	87.80	1.832±0.04
<i>Leptospira</i> 16S rRNA ^e	-	(F) GCGGCGCGTCTTAAACATG (R) TTCCCCCATTGAGCAAGATT	331	86.50	1.849±0.01
<i>Leptospira</i> lfb1 ^f	LA0322	(F) CATTTCATGTTTCGAATCATTTCAA (R) GGCCCAAGTTCCTTCTAAAAG	331	83.40	1.735±0.03
<i>Leptospira</i> LipL32 ^g	-	(F) AAGCATTACCGCTTGTGGTG (R) GAACTCCCATTTCAAGCGATT (P) AAAGCCAG GACAAGCGCCG	242	-	1.788±0.03

^a Accession Number of mRNA sequence in GenBank (NCBI) used for primer design.

^b (F), (R) and (P) indicate forward and reverse primer and probe sequences, respectively.

^c PCR product size in base pairs and melting temperature (Tm) in °C.

^d Efficiency for PCR was determined by elaboration of standard curves as described in Material and Method.

^{e, f, g} See Mérien et al. 1995 [28], Mérien et al. 2005 [14] and Stoddard et al.[15], respectively.