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Differential Response of Cytokines Induced by *Leptospira interrogans*, Serogroup Pomona, Serovar Pomona, in Mouse and Human Cell Lines

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SUMMARY *Leptospira interrogans,* the causative agent of leptospirosis, is an important zoonotic bacterium. The mechanisms and roles of cytokine induction in both humans and animals remain unclear. Therefore, the IFN-γ, IL-6, IL-10 and IL-12 levels were measured by enzyme-linked immunosorbent assay (ELISA) in human THP-1 and mouse RAW 264.7 monocyte cell lines following stimulation with heat-killed *Leptospira interrogans* serogroup Pomona serovar Pomona, *L. biflexa*, *E. coli* or *Salmonella* group B. The production of IL-6 and IL-12 were higher and rose more rapidly in the RAW 264.7 cells with all bacteria. The IL-10 was not detected in the RAW 264.7 cells when induced by leptospires. The IFN-γ level in human peripheral blood mononuclear cells (PBMCs) induced by leptospires was also significantly lower than with other bacteria. When IL-10 and IL-12 mRNA expressions were detected in hamster's spleen, their patterns were similar to what was observed in THP-1 in that IL-12 was only slightly increased while IL-10 expression was high. Moreover, the IFN-γ expression could not be detected in hamsters. The more potent cytokine responses in the RAW 264.7 cells may indirectly reflect the disease outcome in mice which render them to be a good reservoir of leptospirosis. Whether these cytokines have contributed to immunoprotection during the *L. interrogans* infection remains to be further investigated.

Leptospirosis is a worldwide zoonosis caused by pathogenic *Leptospira interrogans*.[1] At present, there are more than 200 serovars of *L. interrogans* that are divided into 11 genomospecies.[2] The clinical manifestations of *L. interrogans* infection range from a flu-like illness to severe and occasionally fatal disease. Worldwide, the annual incidence was increased and varied from 0.1-432.1 per million of population.[3] The wide distribution of leptospirosis results from ability of leptospires to persist in renal tubules of many different wild and domestic animals.[4] These reservoir hosts can shed the organism from their kidneys into urine which can not only infect other animals but also contaminate the environment. Humans are accidental hosts that acquire the organism either by direct contact with urine of infected animals or by indirect contact with contaminated environments. The immunity to leptospiral infection and the pathogenesis of the disease remain poorly understood. Both humoral[5] and cell-mediated

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immunity[6] have been reported to be involved in the defense mechanism. However, the protective host response is thought to depend on circulating antibodies directed against the serovar-specific lipopolysaccharide (LPS).[7] Nevertheless, the mechanisms whereby leptospires can escape the natural defenses of the host, survive and grow in tissues remain to be elucidated. A number of cytokines are involved in the immune response of the host against bacterial infections but little is known about the role of cytokines in leptospiral infection. Pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1, IL-6 and IL-12are able to regulate protective immunity to pathogens and may also involve in induction of immunopathology of the host. TNF-α, IL−12 and IFN-γ levels were found to be elevated in sera of patients with acute severe leptospirosis.[8-10] Although leptospires are extracellular bacteria, IFN-γ was demonstrated to play a protective role in bovines vaccinated with heat killed *L. borgpetersenii* serovar Hardjo[11] whereas the IFN-γ level in patients was found to be very low.[12] In contrast, the TNF-α level was demonstrated to relate to the pathogenesis of leptospirosis.[9] The IFN-γ, IL-12 and TNF-α production were reported to be induced in human whole blood culture by heat-killed leptospires.[13] In the *L. interrogans* infected hamster model, the Th1 cytokine mRNA (TNF-α, IFN-γ, and IL-12), was expressed as early as 1 hour post-infection whereas anti-inflammatory cytokines, such as IL-4 and IL-10, were prominent 1 to 4 days post infection.[14] Since leptospirosis disease progression in human and animals was different, the cytokine responses in the different hosts may reflect the different disease outcome. In this study, the IL-6, IL-10, and IL-12 levels after induction by heat-killed leptospires were therefore monitored at various time intervals by comparing between the monocytes of human and mice which are a non-symptomatic reservoir. Some cytokines (IFN-γ, IL-10 and IL-12) were also observed in the hamster which is a susceptible host similar to humans.[14] As leptospirosis rarely leads to septic shock and stimulates different Toll-like receptors (TLRs) compared to other Gram-negative bacteria such as *E. coli* and *Salmonella* spp.,[15] the induction of the cytokine responses including IL-6, IL-10, IL-12 and IFN-γ by heat-killed *L. interrogans*, serogroup Pomona, serovar Pomona; *L. biflexa* (non-pathogenic) and these other bacteria were therefore compared. This study should lead us to understand more of the immune response that may differ between susceptible and reservoir hosts and also the cytokine profile between leptospires and other Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial cultures

A virulent strain of *L. interrogans*, serogroup Pomona, serovar Pomona isolated from a patient with severe leptospirosis and *L. biflexa* isolated from the environment were used in this study. They were cultured in Neopeptone liquid medium (Difco, USA), enriched with 8% inactivated rabbit serum and incubated at 28-30°C for 3 weeks. The culture was aliquoted and kept at -70°C as a stock. *Escherichia coli* and *Salmonella* group B (isolated from patients in Srinagarind Hospital, Khon Kaen University, Thailand) were cultured in tryptic soy broth. Heat-killed bacteria were prepared by washing and resuspending the bacterial cells in PBS and then boiled for 10 minutes. The viable cells left after boiling were checked before being used in the experiment.

Cytokines induction in cell culture

THP-1 (human monocyte) and RAW264.7 (mouse monocyte) cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum, 25 mM HEPES, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin G and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were passaged to a cell density of 10^6 cells/ml and seeded in 6-well tissue culture plates (Nunc, Roskilde, Denmark). After 24 hour of incubation for confluent monolayer formation, the cells were stimulated with either heat-killed *L. interrogans*, serogroup Pomona, serovar Pomona; *L. biflexa*; *E. coli* or *Salmonella* group B at a bacterium to cell ratio of 100:1 or cells being stimulated with PBS as a control. Supernatants were harvested at 0, 2, 4, 6, 8, 12, 18, 24 and 48 hours after stimulation and assayed for IL-6, IL-12 and IL-10 production by enzyme-linked immunosorbent assay (ELISA).

IFN-γ induction in PBMC

Peripheral blood mononuclear cells (PBMC) were prepared from blood of a healthy donor (with informed consent to donate the blood for laboratory

experiments) by Ficoll-Hypaque gradients and the number of viable cells was determined by trypan blue exclusion. Cells were plated in 6-well tissue culture plates in RPMI 1640 at a concentration of 10^6 cells/ml, then stimulated and harvested the same way as described for monocyte cell lines. The culture supernatants were assayed for IFN-γ production by ELISA.

Cytokine measurement by ELISA

The concentrations of IFN-γ, IL-6, IL-12 and IL-10 in culture supernatants were determined using commercial ELISA kits (Endogen, Pierce Biotechnology, Inc, IL., USA) according to the manufacturer's instructions. Samples were assayed in duplicate and all experiments were performed at least twice. The result was expressed as mean values and the statistical analysis was performed by the independent Student t test to compare between *E. coli* and *Salmonella* group B, *L. biflexa* or *L. interrogans*, serogroup Pomona, serovar Pomona.

***In vivo* infections**

Three to 4 week old (approx. 40 to 59 g) Syrian golden hamsters (*Mesocricetus auratus*) that had been bred and housed under hygienic conventional conditions at the Animals Laboratory Unit, Faculty of Medicine, Khon Kaen University, were used. All animal experiments received an approval from the animal ethics committee, Khon Kaen University.

Twenty hamsters were injected intraperitoneally with 50 LD_{50} doses of viable *L. interrogans*, serogroup Pomona, serovar Pomona (ca. 1.5 x 10^4 cells) and 4 hamsters were injected with PBS to serve as a control. Four infected hamsters at a time were euthanized on days 0, 1, 3, 5 and 10 after infection to collect their blood from the retroorbital plexus to confirm the presence of *L. interrogans* by culture and PCR amplification.[13] After their blood was taken, their spleens were also aseptically taken and immediately submerged in RNA later® (Ambion, USA), and placed on ice to prevent RNA degradation.

RNA extraction

Spleen (~50-100 mg) was dispersed in 1 ml of Trizol reagent (Invitrogen, Life technologies, USA) using glass tissue homogenizers. The supernatant was collected to a new tube after centrifugation at 12,000 x *g* for 10 minutes and mixed well with 200 μl chloroform. The aqueous phase was removed by centrifugation at 12,000 x *g* for 15 minutes and 500 μl isopropyl alcohol was added to precipitate the RNA. The RNA pellet was washed with 75% ethanol and re-suspended in 30 μl of RNase-free water. Contaminated chromosomal DNA was removed by digestion with RNase-free DNases (Promega, USA) at 37°C for 30 minutes. The enzyme activities were destroyed by heating at 65°C. Total RNA was quantified by spectrophotometry (Altrospec, USA) at 260 and 280 nm. A 260/280 ratio of greater than 1.8 was considered acceptable.

Semi-quantitative RT-PCR

RT-PCR amplification of IFN-γ, IL-10 and IL-12 was performed in a single tube using a SuperScript™ One-Step RT-PCR kit with Platinum® *Taq* (Invitrogen, Life technologies, USA) according to the manufacturer's instructions. Hypoxanthine-guanine phosphoribosyl transferase (HPRT), a house keeping gene, was used as an expression reference and positive control. The RNA extracted from Concanavalin A stimulated THP-1 was used as the cytokines mRNA expression positive control. The template quantity and cycle of amplification were titrated and optimized to receive a mid-log amplification using a Thermol Cycler 2400 (Perkin-Elmer, USA). Three micrograms of total RNA were reverse transcribed and amplified in a final concentration of 1x reaction buffer (containing 0.2 mM of each dNTP, 1.2 mM $MgSO_4$), 2 mM $MgSO_4$, 1 μM each of sense and anti-sense primers, and 1 U of RT/*Taq*. The reverse transcription step was performed at 45°C for 30 minutes and a pre-denaturation step was done at 94°C for 2 minutes. The conditions for amplification were as follows: 35 cycles with denaturation at 94°C for 50 seconds, primer annealing for 1 minute at 48°C for HPRT and IL-10, 42°C for IL-12 and 50°C for IFN-γ, primer extension at 72°C for 50 seconds and a final cycle at 72°C for 7 minutes. Sequences of oligonucleotide primers specific for HPRT, IFN-γ, IL-10 and IL-12p40 genes (Table 1) were designed using published Syrian golden hamster cDNA sequences[16] by GeneFisher primer design software.[17] The RT-PCR amplification products were resolved by electrophoresis on 2.0% agarose

gels and visualized under UV light after staining with ethidium bromide.

The intensity of RT-PCR amplicons was evaluated using Image Master 1D gel electrophoresis software (Amersham Pharmacia Biotech, USA). The output values were expressed as the ratios of each cytokine band's intensity to the housekeeping gene (HPRT) intensity which then was presented as mean-fold-increase of each cytokine ratio each day post-infection.

RESULTS

In THP-1 cell line, the heat-killed *L. interrogans*, serogroup Pomona, serovar Pomona; *L. biflexa*; *E. coli* and *Salmonella* group B stimulated the production of IL-6 and IL-12 in a similar pattern. The production levels of these cytokines increased slightly over the first 24 hours and then more rapidly at 48 hours after stimulation (Fig. 1A, 1B). The IL-12 level stimulated by *E. coli* was similar to that of *L. interrogans* but significantly lower than *Salmonella* group B. At 48 hours after stimulation, *L. interrogans* induced significantly lower IL-6 than that of *E. coli*. The levels of IL-10 production in all groups were not high when compared to other cytokines (Fig. 1C). However, *L. interrogans* provoked significantly lower IL-10 levels when compared to *E. coli* while *Salmonella* group B stimulated a significantly higher response. *L. biflexa* stimulated all cytokines in a similar pattern as pathogenic leptospires.

On the other hand when looking at RAW 264.7 cells, even though they showed similar patterns of IL-6 and IL-12 production among bacteria tested, they markedly increased as early as 4 and 6 hours after stimulation (Fig. 1D and 1E). Moreover, their levels were also 10 times higher than that observed in THP-1 cells. These results indicated the difference in responses between human and mouse cell lines to all bacteria tested. Interestingly, the level of IL-10 could not be detected when RAW 264.7 cells were stimulated with either heat killed *L. interrogans* or *L. biflexa* (Fig. 1F).

In human PBMCs, the levels of IFN-γ production stimulated by heat-killed *L. interrogans* was significantly lower when stimulated by either *E. coli* or *Salmonella* group B (Fig. 2).

When cytokine mRNA expression was detected in hamster spleens, the IFN-γ expression was undetectable while IL-12 was slightly increased to peak quite late on day 3. Interestingly, the IL-10 expression was greatly increased immediately after infection similar to what was observed in the THP-1 cell line (Fig. 3).

DISCUSSION

The importance of antibody-mediated immunity in protection against *L. interrogans* infection has been clearly demonstrated.[5,18,19] The role of T cells and cytokines involved during infection, however, is not clear and may also be important for protection and pathogenesis of the organism. *L. interrogans* or its components were reported to induce cell activation including nitric oxide[20,21] and cytokine production,[13,22] up-regulation of adhesion molecules

Table 1 The specific primer sequences and the sizes of the PCR products

Target	**Oligonucleotide sequence** (5'-3')	**Product size** (bp)
HPRT	GCGATGTCATGGTAGAGA	110
	GGGAGTGGATCTATCACA	
IFN-γ	TCCTATCGCGTTGGCCTA	497
	TCCACCCCCAAAACAGCA	
IL-10	CATGCTCCGAGAGCTGA	239
	CTGCAGTTGCCTCCTGA	
IL-12p40	CTCTGAGCCACTCACGA	151
	GTCAGTGCTGATTGCA	

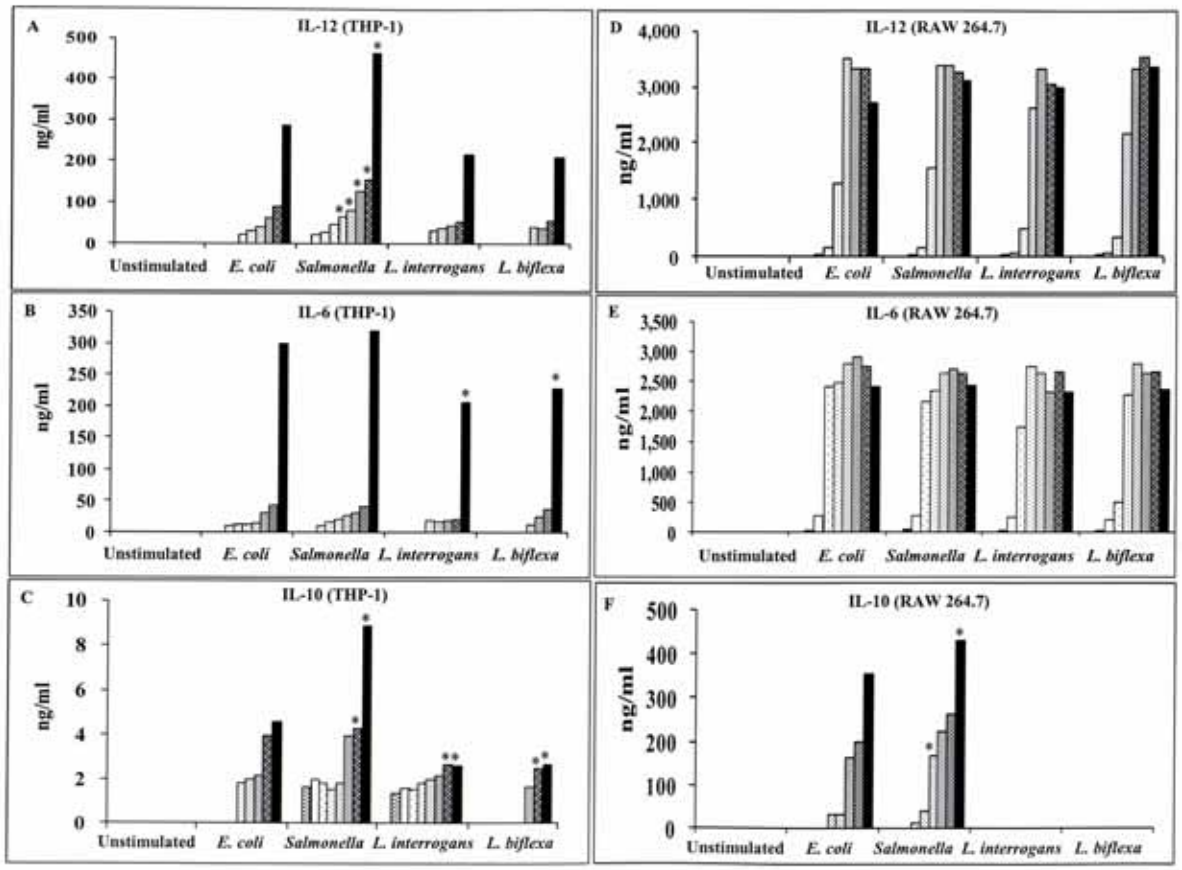

Fig. 1 Kinetics of cytokine production by THP-1 (A, B and C), RAW 264.7(D, E and F) in response to heat-killed *Leptospira interrogan*, serogroup Pomona, serovar Pomona; *L. biflexa*; *E. coli* and *Salmonella* group B for 0 (▤), 2 (□), 4 (⁙), 6 (⁙), 8 (▨), 12 (▩), 18 (▩), 24 (▩), and 48 (■) hours. The supernatants of stimulated cells were examined for IL-12 (A and D), IL-6 (B and E) and IL-10 (C and F) by ELISA. Each bar represents the mean for duplicate samples. The asterisks (*) indicate cytokine production significantly different from *E. coli* at the same interval time ($p < 0.05$).

in many kinds of cells[23] and apoptosis of lymphocytes.[17,19-21] Several *L. interrogans* cellular components such as lipoproteins, lipopolysaccharide (LPS) and glycolipoprotein (GLP) are toxic and may participate in the pathogenesis of the disease.[24,25] GLP is reported to stimulate the human PBMCs to produce TNF-α, IL-10 and IL-6.[20,24] While the disease in humans is associated with jaundice and pulmonary hemorrhage, the infected mouse has no symptoms and becomes a good reservoir host. The high secretion of pro-inflammatory cytokine levels such as TNF-α, and IL-6 from mouse macrophages was correlated with the bacterial clearance whereas the TLR4 knockout mice that gave lower cytokine levels showed a high bacterial load in the liver, lung and kidney with disease manifestations similar to humans.[26] This evidence may indicate the differences in the immune response in these hosts. In the current study, we first demonstrated the differences in cytokine responses in human and mouse monocyte cell lines and also in human PBMCs induced by *L. interrogans* when compared to *E. coli* and *Salmonella* group B.

The IL-12 was demonstrated to be stimulated by whole leptospiral cells while GLP was found to play a role in IL-6 production.[13,27] The production of IFN-γ elicited by *L. interrogans* appeared to largely depend on the release of IL-12.[13] The cells that have been shown to be responsible for the pro-

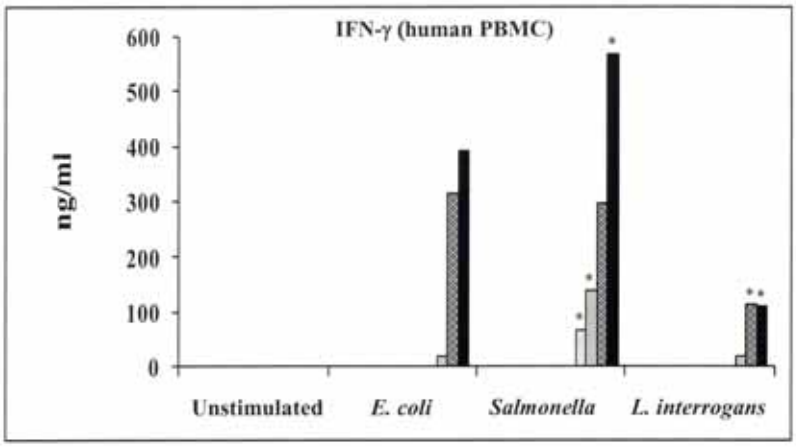

Fig. 2 Kinetics of IFN-γ production by PBMC in response to heat-killed *L. interrogans*, serogroup Pomona, serovar Pomona; *E. coli* and *Salmonella* group B for 0 ( ), 2 ( ), 4 ( ), 6 ( ), 8 ( ), 12 ( ), 18 ( ), 24 ( ), and 48 ( ) hours as determined by ELISA. Each bar represents the mean for duplicate samples. The asterisks (*) indicate cytokine production significantly different from *E. coli* at the same interval time ($p < 0.05$).

duction of IL-12 are γδT cells.[28] In this study, we found both human and mouse monocytes could express high levels of IL-12. IL-6 is a pleiotropic cytokine influencing antigen-specific immune responses and inflammatory reactions. It is a potent inducer of the acute-phase protein response during inflammation.[29] Our results demonstrated that leptospiral whole cells, either pathogenic or non-pathogenic, were less able than *E. coli* (extracellular bacteria) or *Salmonella* group B (intracellular bacteria) in stimulation of IL-12 or IL-6 expression in human cell lines (Fig. 1, 48 hours). This may be due to the antigenic differences mostly in the LPS component from *E. coli* and *Salmonella* group B. Moreover, it has been shown that leptospiral LPS uses TLR2 instead of TLR4 for signaling in human cells.[15] However, heat stable molecules other than leptospiral LPS play a role in stimulation of mouse macrophage IL-6.[26] The effect of all the heat-killed bacteria tested was the same when they were used to stimulate RAW264.7 cells. These results indicated the differences of cytokine responses between human and mouse cells and whether these variations play a role in the contradiction of the disease profiles or pathogenesis remains to be investigated. The dissimilarity in these responses was reported to be due to the differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells.[30] The TLR4 of the mouse macrophage, however, is mediated by a lipid A other than LPS and was reported to protect

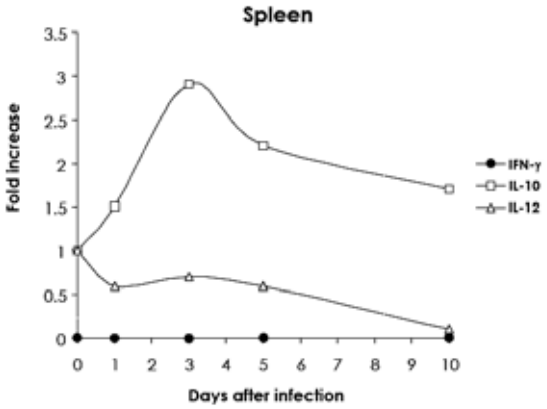

Fig. 3 Kinetics of cytokine mRNA expression in spleens taken from hamsters infected with viable *Leptospira interrogan*, serogroup Pomona, serovar Pomona. Total RNA from each spleen was amplified by quantitative RT-PCR. The band intensities were quantified by image analysis software. Data are presented as the fold-increase of the cytokine ratio in infected hamsters compared to uninfected controls.

against lethal *L. interrogans* serovar Icterohaemorrhagiae infection. It appears to contribute to *in vivo* control of the leptospiral burden[26] in that it did not activate human macrophages.[30] A previous study using leptospiral LPS showed that TLR2/TLR1 were the predominant receptors in human cells, whereas

TLR2 and also TLR4, contributed to activation in murine cells.[30]

Among the bacteria studied, our experiments also showed the difference in IL-10 induction between human and mouse monocytes. The RAW 264.7 cell lines showed no IL-10 responses after stimulation by leptospires but produced a low level in THP-1 cells. The *in vivo* study conducted in the Syrian Golden hamster showed the increased in IL-10 level immediately after infection. These data indicated indirectly that some heat labile components from the living organisms may be involved in IL-10 stimulation. IL-10 is an anti-inflammatory cytokine that suppresses the proliferation and cytokine expression of Th1 cells. The progressive decrease of IL-12 in hamster spleens after infection may be due to the inhibitory effect of IL-10. In contrast, the high level of IL-10 in leptospirosis patients was proposed to be associated with less severe disease and survival.[31] Thus, all observations confirmed that the differential cytokine responses led to the diverse disease outcome. In leptospirosis, there are very rare cases of septic shock and its LPS has been reported to be different from LPS of other Gram-negative bacteria.[24] This observation was confirmed by the low IFN-γ levels produced by human PBMCs when induced with *L. interrogans* and compared with *E. coli* and *Salmonella* group B (approximately 4-6 times lower; Fig. 2) and undetectable in hamster (Fig. 3). The plasma IFN-γ levels in leptospirosis patients were low and indistinguishable from local healthy controls[11] which was different from the high level rapidly within a few hours[14] and then decreased to an undetectable level in days in the hamster with leptospirosis (Fig. 3). The IFN-γ was demonstrated to associate with the protection in the bovine leptospiral vaccine against *L. borgpetersenii* serovar Hardjo.[12] The diverse host responses may indirectly point to the disease outcome. Our preliminary results (unpublished data) also indicated that LPS of *L. interrogans*, serogroup Pomona, serovar Pomona, was a poor IL-6 stimulator.

In conclusion, we demonstrated the differences in cytokine responses between human and mouse cell lines that may reflect the disease outcome as the mouse is known to be a maintenance host of leptospirosis. Moreover, the cytokine induction in THP-1 cell line and IFN-γ in human PBMCs by leptospires is lower when compared to other Gram-negative bacteria. The mechanisms and roles of these cytokines in the protection of different hosts during *L. interrogans*, serogroup Pomona, serovar Pomona infection need to be further investigated.

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