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Evaluation of Th1 and Th2 immune response in clinical and sub-clinical scrub typhus infection

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ABSTRACT

Scrub typhus (ST), caused by a gram negative intracellular bacteria– *Orientia tsutsugamushi,* is one among the leading causes of febrile illness across Southeast Asia, including India. Clinical presentation can vary from asymptomatic to severely fatal. Th1-cell mediated immunity has been suggested to play an important role against ST infection in animal models. However, human data on protective immunity are limited. The present study was undertaken to identify host immune correlates that could confer protection in individuals that remain clinically asymptomatic/sub-clinical. Serum cytokine profiling and mRNA expression levels of Th1 (TNF-α, IFN-γ, IL-2) and Th2 (IL-10, IL-6, IL-4) cytokines was studied amongst the clinical and sub-clinical infections. It was observed that a Th1/Th2 pattern is not involved in human ST infection irrespective of being a symptomatic or asymptomatic presentation. However, significant difference was observed in IL-10 serum and gene expression levels. This study suggests a possible role of IL-10 in disease phenotypic presentation. Over-production of IL-10 was found to be a significant factor contributing to the severity of the disease whereas a protective immune mechanism might exist with a low level of IL-10 in ST infection.

1. Introduction

Scrub typhus (ST) is an infectious bacterial diseases caused by an intracellular gram negative bacteria-*Orientia tsutsugamushi,* transmitted by the bite of larval (chigger) mites [1]. The disease has been endemic to a part of the world known as the 'tsutsugamushi triangle' [2]. However, recent studies have revealed the presence of the disease beyond this area, suggesting the disease to have a global impact [3,4]. Globally, about one billion people are at risk of getting infected annually with mortality ranging from 0 to 70% in untreated cases [5,6]. There is no licensed vaccine for ST. Efforts of developing an effective vaccine have been hampered due to the complex antigenic heterogenicity of the bacterial strains [1,5,7]. Clinical presentation in ST varies from remaining asymptomatic to severely fatal [5]. Severity depends on the virulence capacity of the infecting strain; with studies in mice models suggesting 'Karp' like strain to be highly pathogenic and Shimokoshi as the least severe one [5,8]. However, data on human virulence remains inconclusive. Beside the virulence of the pathogen, host immune responses are crucial in determining the outcome of a disease [5]. Immunity against ST is complicated due to the pathogen antigenic diversity, failure to provide cross protection against divergent strains as well as weak homologous protection [5,9].

Cell mediated immunity plays an important role against ST infection. Hyper-cytokinemia has been determined to be an important factor responsible for development of symptoms in infected patients [10–15]. Experimental studies in mice models have suggested that protective immunity to ST is mediated by T-helper (Th)-1 cells, as demonstrated by adoptive transfer of antigen-specific IFN-γ-producing T cell [16]. It is well known that Th1 immune responses are crucial to protection against intracellular pathogens [17]. However, inclination towards a Th2 lineage like elevated IL-6, IL-10 has also been variably observed in ST infection. Thus, there seems a concurrent expression of CD4 + Th- 1 and Th-2 cytokines in ST patients. It is likely that commitment towards a particular lineage or a homeostatic balance of Th-subsets might be an important factor to look for the disease differential phenotypic presentation. Keeping this in consideration, we evaluated the cytokine

Abbreviations: ST, Scrub typhus; TNF-α, Tumor necrosis factor-alpha; IFN-γ, Interferon-gamma; IL, Interleukin; CXCL, Chemokine (C-X-C) ligand; CCL, Chemokine (C-C) ligand; Th1, T-helper 1; Th2, T-helper 2; EDTA, Ethylene-diamine-tetra-acetic Acid; ELISA, Enzyme-linked immunosorbent assay; DHR, Department of Health Research; cDNA, complementary Deoxyribonucleic acid; RNA, ribonucleic acid; mRNA, messenger ribonucleic acid; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative polymerase chain reaction; IQR, Inter-quartile range; MAIT, mucosa associated invariant T-cells; NO, Nitric oxide

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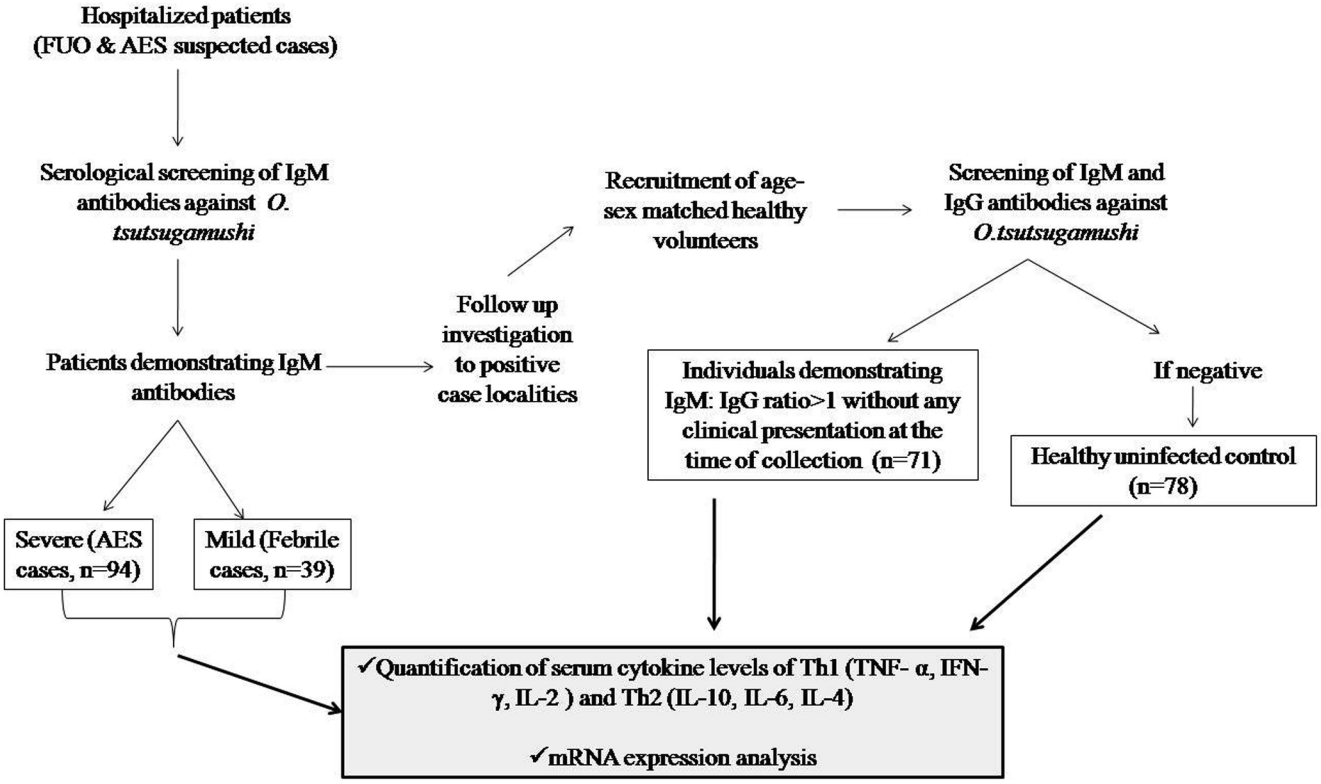

Fig. 1. Work flow of the study.

profiling of Th1 (TNF-α, IFN-γ, IL-2) and Th2 (IL-6, IL-10, IL-4) cytokines in ST infected patients and compared with the asymptomatic sub clinical infections.

2. Material and methods

2.1. Study groups

This study comprised of 204 individuals (133 symptomatic cases and 71 asymptomatic controls) (Fig. 1). Patients presenting with fever as a primary symptom with or without an eschar (a necrotic lesion formed at the site of mite bite), attending Assam Medical College & Hospital, Dibrugarh, Assam were recruited as cases. Diagnosis was based on serological evidence of IgM antibodies against ST using Scrub typhus IgM Detect ELISA assay (InBios International, USA). An epidemiological cut off of 0.5 was considered [18,19]. Symptomatic cases were further stratified as mild and severe based on clinical symptoms. Cases presenting with acute fever, headache, bodyache, vomiting, and nausea were considered as mild (n = 39) ST cases. Patients diagnosed as an acute encephalitis syndrome (AES) case by the clinicians were considered as severe cases (n = 94). Age-sex matched healthy volunteers were recruited during retrospective follow up of clinical cases. Asymptomatic individuals demonstrating IgM:IgG ratio $>$ 1, indicative of an acute infection were considered as asymptomatic (n = 71). Inclusion criteria for asymptomatic subjects also included absence of prolonged fever ($\geq$5days) during the past three months from the date of collection. Another 78 samples from healthy uninfected volunteers was also used in the analysis. Epidemiological cut-off of 0.5 for IgM and IgG was considered based on the baseline titre among the healthy population of this region. IgG antibodies against ST were detected by using Scrub typhus IgG Detect ELISA assay (InBios International, USA). Informed written consent was taken from all enrolled participants. Blood samples were collected in two vials- one in EDTA vial (for nucleic acid extraction) and the other in plain vial (for separation of serum for antibody detection and cytokine level quantification). DHR guidelines for rickettsial disease diagnosis were followed to rule out cross reactivity with other similar febrile illness [18].

2.2. Quantification of Th1 and Th2 serum cytokine level

Serum samples were subjected for quantifying circulating serum levels of Th1 pro-inflammatory cytokines *viz*., TNF- α, IFN- γ, IL-2 and Th2 anti-inflammatory cytokines *viz*., IL-10, IL-6, IL-4 using commercially available cytokine ELISA kits: Human TNF-α ELISA set- BD OptEIA, Human IFN-γ ELISA set-BD OptEIA, Human IL-6 ELISA set-BD OptEIA, Human IL-10 ELISA set-BD OptEIA, Human IL-2 ELISA set-BD OptEIA, Human IL-4 ELISA set- BD OptEIA (BD Biosciences, USA). Tests were performed as per manufacturers' instruction. The absorbance of standards, controls and samples was calculated by subtracting the zero standard absorbance from each. A standard curve was prepared by plotting the cytokine concentration on the X-axis and absorbance read on the Y-axis. Best fit curve was drawn through the standard points. Concentrations of unknown samples were determined by plotting against the standard curve. The lowest limit of detection for TNF-α, IFN-γ, IL-10, IL-6, IL-2 and IL-4 were 2 pg/ml, 1 pg/ml, 2 pg/ml, 2.2 pg/ml, 1 pg/ml and 2 pg/ml respectively.

2.3. Relative quantification

ST ELISA positive samples of each group were subjected for mRNA expression of the targeted cytokines (TNF-α, IFN-γ, IL-10 and IL-6). Uninfected healthy control samples (n = 40) were used as a calibrator in the experiment for each gene. Whole blood samples were used for extraction of RNA using the Qiagen RNA blood mini kit (Qiagen, Hilden, Germany) as per the manufacturers' instruction. Attempts were made to extract RNA on the same day of collection to minimize/avoid RNA degradation. cDNA synthesis was performed from extracted RNA using Qiagen Reverse transcriptase cDNA synthesis kit (Qiagen, Hilden, Germany). Validated primers from Qiagen Quantitect primer assay (Qiagen, Hilden, Germany) for each of the cytokines was used for

mRNA expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization [20,21]. qPCR was performed using the Quantitect SYBR green method (Qiagen, Hilden, Germany). Thermal cycling condition included initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s and extension at 72 °C for 20 s. Amplification upto 35th cycles were considered as positive for the targeted gene. Post amplification, melting curve analysis was performed to verify the specificity of the assay for each gene. Each sample was assayed in duplicate and each target gene was normalized to the gene expression of GAPDH. Relative quantification was calculated using the Livak or $2^{-\Delta\Delta CT}$ method [22].

2.4. Statistical analysis

Statistical analysis was performed employing SPSS version 20. Log transformed values were used for representing serum cytokine levels. Group comparison was initially done by the non-parametric Independent samples Kruskal Walis test. When significant difference was indicated, further groups were compared by the non-parametric Mann-Whitney test with log transformed values. Binary logistic regression was performed to ascertain the effect of age, gender and individual cytokines. Spearman rank correlation coefficient was used to determine the correlation between circulating serum cytokine level and their gene expression. A $p < 0.05$ was considered as statistically significant for all the statistical analysis.

3. Results

Demographic characteristics reveal predominance of males in all the study groups. Laboratory parameters of clinical cases are summarized in Table 1. The mean age of severe cases, mild and asymptomatic were 33.39 ± 17.24, 34.08 ± 15.23 and 33.61 ± 15.48 respectively. No significant difference observed in the gender and mean age amongst the groups (Table 2).

3.1. Serum protein levels

Among the six studied cytokines, serum concentration of IL-10 and IL-6 was found to be significantly elevated among the cases compared to the asymptomatic controls. IL-10 was significantly high among the symptomatic cases (mild: 2.02 pg/ml (median); Inter-quartile range (IQR):1.8–2.1 *Vs* severe: 2.07 pg/ml; IQR: 1.91–2.16) when compared to the asymptomatic control group (1.79 pg/ml; IQR: 1.61–1.94) with $p < 0.0001$ and $p < 0.0001$ respectively. For IL-6 too, elevated levels in serum concentration among the symptomatic subjects (mild: 2.1 pg/ml; IQR: 1.63–2.5 *Vs* severe: 2.05 pg/ml; IQR: 1.69–2.31) was found to be significantly higher compared to the asymptomatic controls (1.82 pg/ml; IQR: 1.42–2.1) with $p < 0.05$ (Fig. 2). For both IL-10 and IL-6, we did not observe significant difference between mild and severe ST cases. For the rest of the cytokines (IFN-γ, TNF-α, IL-2, IL-4), we did not observe any significant difference. IL-4 was below the limit of detection in majority of the positive cases, irrespective of being symptomatic or asymptomatic.

When compared with healthy uninfected controls, significantly higher levels of TNF-α serum concentration was noted in both cases (severe & mild) and asymptomatic controls. For the rest of the cytokines, serum levels of IL-10 were significantly higher among the cases ($p < 0.0001$), whereas difference in the asymptomatic controls were insignificant compared to the healthy uninfected controls (Fig. 2). For IFN-γ, IL-6, IL-2 and IL-4 we did not observe any significant difference with the healthy uninfected control.

Table 1
Laboratory findings of clinical cases.

Characteristics	Severe cases (n = 83)	Mild cases (n = 33)
WBC count (/mm^3)	10,547 (4657.37)	10,000 (4383.49)
Hb (g/dl)	10.82 (2.01)	11.35 (1.96)
AST (IU/L)	116.93 (163.75)	143.6 (95.11)
ALT (IU/L)	96.18 (89.74)	151.2 (133.89)
ALP (IU/L)	166.12 (131.12)	329.75 (365.97)
Urea (mg/dl)	46.72 (43.64)	67.57 (95.96)
Creatinine (mg/dl)	1.21 (1.18)	1.9 (1.7)
Serum Sodium (mmol/L)	134.7 (8.71)	135.66 (8.73)
Serum Potassium (mmol/L)	3.9 (0.79)	3.8 (0.435)
Albumin (g/dl)	2.84 (0.914)	3.4 (0.61)
Globulin (g/dl)	5.97 (8.67)	3.6 (0.99)

Data are expressed as mean (Standard deviation)
AST: Aspartate Aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase

3.2. Gene expression

Fold change difference among the cases and asymptomatic controls was ascertained for TNF-α, IFN-γ, IL-10 and IL-6 by comparing to the healthy uninfected controls. IL-10 in severe cases was expressed at 18.41-fold, mild cases at 19.58-fold and asymptomatic controls at 5-fold higher than the healthy uninfected control group. IL-6 was expressed at a difference of 4.86-fold (severe cases), 3.85-fold (mild cases) and 5.19-fold (asymptomatic controls) compared to the healthy uninfected control. mRNA levels of IL-10 was seen to be significantly different between the study groups ($p < 0.05$) whereas difference in IL-6 was insignificant. For TNF-α and IFN-α, so significant difference was found between cases and asymptomatic controls (Fig. 3).

3.3. Association of serum protein levels with their mRNA levels

Correlation analysis of mRNA levels with the circulating serum levels showed a positive correlation for IL-6 serum and gene expression levels among cases only ($\rho = 0.357$, $p < 0.05$). No significant correlation for IL-10 was observed in any group. A binary logistic regression was performed to ascertain the effect of age, gender and individual cytokines on the likelihood of developing into a symptomatic case. No significant effect of age and gender was noted. However, an increasing IL-10 was found to be associated with an increasing likelihood to develop into a symptomatic case ($p < 0.05$).

4. Discussion

T-cell mediated immunity is believed to be an important defense mechanism against rickettsial infections, including ST [23–25]. The difference in clinical presentation and severity of a disease depends on the host immunity apart from the virulence capacity of the infecting pathogen [5]. Our study reveals a possible role of IL-10 in phenotypic disease presentation in ST infection. Experiments in mice models had earlier suggested a role of Th1 cells in inducing protective immunity to ST [16]. We observed that a typical Th1/Th2 pattern is not followed in human ST infection, irrespective of being a symptomatic or asymptomatic. Similar results have also been observed by Chung *et al* [12].

TNF-α, a well characterized pro-inflammatory Th1 cytokine elicits efficient host defense against a number of intracellular pathogens [26]. In ST infection, elevated level of TNF-α has been emphasized to contribute in disease progression [10,11]. A significant association between TNF-α serum level with disease severity has been observed during the acute phase infection [11]. Contrastingly, an impaired TNF-α production by mucosa associated invariant T-cells (MAIT) has been suggested to play a vital role in ST pathogenesis via a negative feedback mechanism [27]. Our study observed elevated serum TNF-α in both symptomatic and asymptomatic infections ($p < 0.0001$).

Counterbalancing of TNF-α mediated by the anti-inflammatory cytokine IL-10 is a well established phenomenon [28,29]. An in-vivo experimental study on mice revealed *O. tsutsugamushi* infection to

Table 2
Characteristics of the study groups.

Characteristic	Severe cases (n = 94)	Mild cases (n = 39)	Asym. control (n = 71)	Healthy control (HC)(n = 78)	p-value Severe *Vs* Mild	p-value Severe *Vs* Asym.	p-value Mild vs Asym.	p-value HC *Vs* Severe	p-value HC *Vs* Mild	p-value HC *Vs* Asym.
Demographic										
Age (Mean ± Standard Deviation)	33.39 ± 17.2	34.08 ± 15.2	33.61 ± 15.4	32.36 ± 14.6	NS[a]	NS	NS	NS	NS	NS
Gender (Male/Female)	60/34	25/14	39/32	40/38	NS	NS	NS	NS	NS	NS
Serum protein level[#]										
TNF-α	1.94 (1.69–2.05)	2. 0 (1.78–2.07)	1.9 (1.03–2.11)	1.45 (1.16–1.72)	0.442	0.292	0.265	< **0.0001**[*]	< **0.0001**[*]	**0.014**[*]
IFN-γ	1.63 (1.35–1.89)	1.7 (1.37–1.91)	1.64 (1.34–1.84)	1.65 (1.28–1.82)	0.6	0.705	0.364	0.374	0.205	0.635
IL-10	2.07 (1.91–2.16)	2.02 (1.8–2.1)	1.79 (1.61–1.94)	1.72 (1.64–1.85)	0.223	< **0.0001***	< **0.0001***	< **0.0001***	< **0.0001***	0.167
IL-6	2.05 (1.69–2.31)	2.1 (1.63–2.5)	1.82 (1.42–2.1)	1.94 (1.69–2.1)	0.619	**0.011***	**0.03***	0.097	0.114	0.195
IL-2	0.75 (0.75–0.93)	0.9 (0.74–1.29)	0.8 (0.57–0.96)	0.77 (0.47–1.04)	NS	NS	NS	NS	NS	NS
IL-4	NC[b]	NC	NC	1.25 (1.13–1.53)	–	–	–	–	–	–

TNF-α: Tumor Necrosis factor-alpha; IFN-γ: Interferon-gamma; IL: Interleukin
[*]significance by non-parametric Independent samples Mann-Whitney test
[#]Data expressed as median (Inter-quartile range)
[a]Not significant
[b]Not calculated

suppress TNF-α production by inducing IL-10 secretion. IL-10, often known as the master regulator of the immune system is known for its immunosuppressive properties resulting in an equilibrated immune response leading to successful bacterial clearance and recovery [30]. However, excessive production of this cytokine has shown detrimental effects, failing to control the disease progression in many bacterial infections [31]. We found elevated levels of circulating IL-10 in serum as well as in the gene expression level amongst the symptomatic patients compared to the asymptomatic infections ($p < 0.0001$). An increasing likelihood to develop into a symptomatic case with an increase in IL-10 secretion was also found ($p < 0.001$). On the other hand, we did not find any significant difference between the asymptomatic and healthy uninfected controls, which suggests that a protective immune mechanism may exist with absence/low levels of IL-10 in ST infection. The opposing effect of IL-10 was recently suggested in driving excessive suppression of the host immune response in infection with intracellular bacteria which enhances bacterial dissemination and persistence within the host rather than its clearance [31]. Highly virulent intracellular pathogens have been suggested to stimulate IL-10 production for facilitating their survival and persistence within the host [32].

Previous studies have suggested IL-10 and IL-6 as active inducer towards a Th2 lineage. IL-6 is known to promote IL-4 secretion further enhancing Th2 mediated immune response [33]. However, IL-4 does not seem to be contributing in human ST infection, as this remained undetected in majority of our study subjects. Hence, commitment towards a Th2 lineage remains questionable. Despite this, we have observed that both IL-10 and IL-6 serum concentration were significantly higher among the symptomatic cases. Recent studies have provided good evidence of IL-10 and IL-6 secretion from macrophages, dendritic cells and even T-regulatory cells beside the Th2 cells [30]. This possibly suggests the combinatorial over expressive effect of IL-10 and IL-6 secreting immune cells towards ST disease pathogenesis. Interestingly, only IL-10 was found to be expressed at a significantly higher fold difference in the symptomatic cases. IL-6 did not show any significant difference in the expression levels. Whether the absence or the low levels of IL-10 is beneficial to the host in ST infection could also likely depend on the infecting pathogens' virulence, infection dose, genetic predisposition, etc. It is difficult to conclude as multiple strains of *O. tsutsugamushi* with differential virulent capacity may coexist in the same geographical area [5]. Our previous studies have identified the circulation of multiple strains of *O.tsutsugamushi* in Assam [19]. However, the virulence capacity and bacteraemia in the study cases was not determined in the present study. This is a limitation in our study. In-vitro studies have indicated infection dose dependent secretion of IL-10 in *O. tsutsugamushi* infection [34]. High levels of IL-10 have been found to be produced in response to low infectious dose, whereas during high infectious dose, high levels of TNF-α was seen to be induced by the macrophages. Additionally, human bacteraemia was shown to be differentially related to pro-inflammatory cytokines and IL-10 suggesting both to be induced by different constituents of *O. tsutsugamushi* bacteria [13]. The present study was also based on one time point collection; hence, the convalescent scenario of IL-10 was not studied. Further in-vitro and in-vivo studies will enhance our understanding on this aspect. Nevertheless, elevated IL-10 and IL-6 protein levels have been found to contribute to the severity and disease presentation. A study conducted on Indian population identified a distinct cytokine profile in ST patients that differed from healthy as well as other infectious disease control [15]. Their study also showed higher levels of IL-10 in ST patients than other infectious disease control. Our study suggests secretion of IL-10 to be differentially expressed in clinical and sub-clinical ST infection. Apart from IL to 10, IL-8 was identified to have a significant impact on disease severity by Astrup et al. [15]. Unfortunately, due to sample exhaustion, we could not test our samples for IL-8. However, it would be interesting to study the scenario of IL-8 in sub-clinical infections.

Doxycycline, a broad spectrum antibiotic having anti-inflammatory properties is the current drug of choice for treating scrub typhus [35]. Some studies have revealed that Doxcycline induced anti-inflammatory properties have no effect on IL-10 secretion [36]. Rather, Doxcycline acts on the expression of inflammatory mediators and does not modify or lower the IL-10 levels [36]. Another study has found that Doxycycline reduces mortality by reducing Nitric oxide (NO) via an IL-10 independent mechanism in some bacterial infections [37]. In *O. tsutsugamushi* infection, NO activates pathways that enhance the growth of the pathogen [38]. With the current advent of reports of Doxycycline

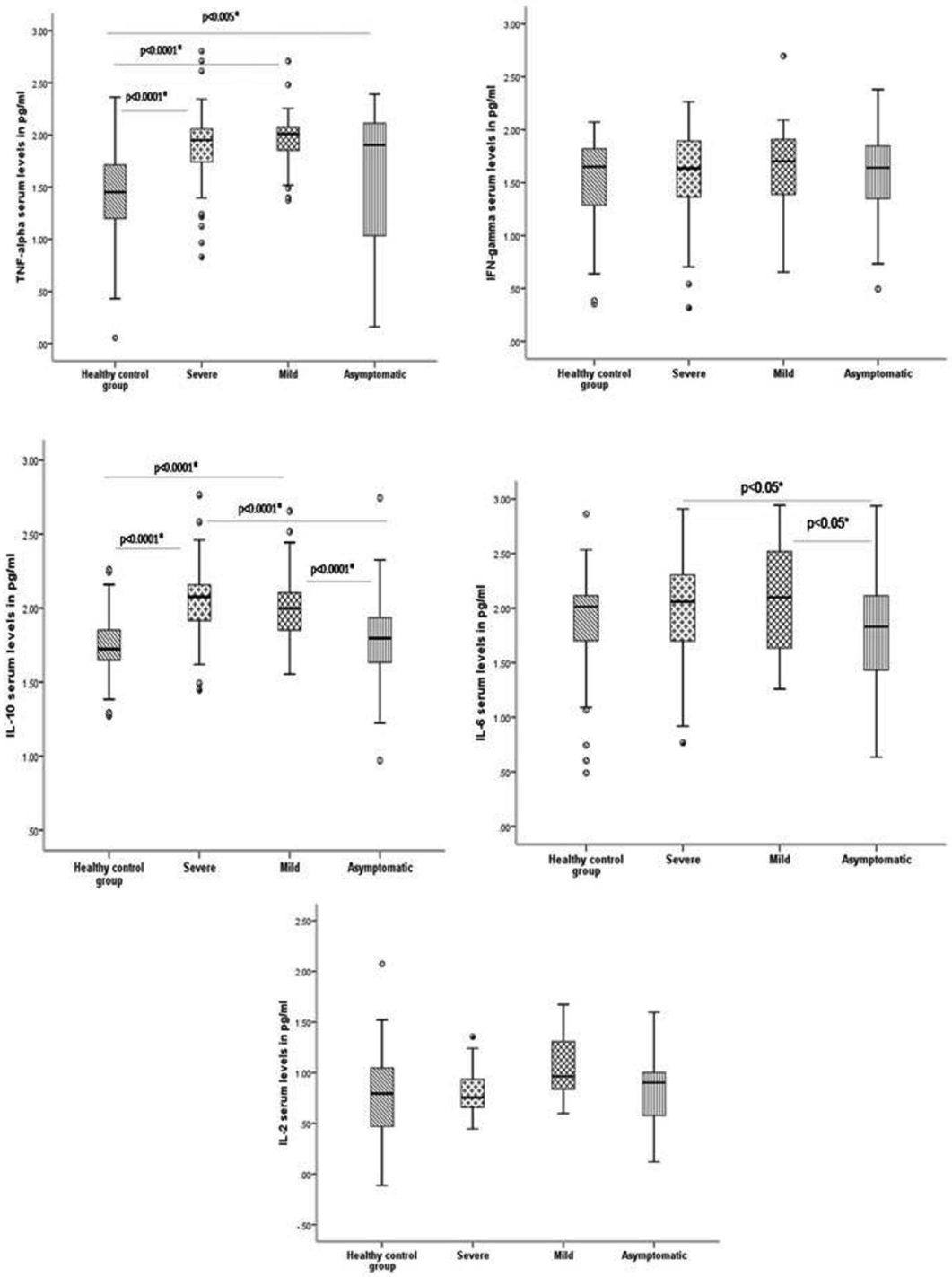

Fig. 2. Serum levels of TNF- α, IFN-γ, IL-10, IL-6. Data are represented in box plots prepared in SPSS version 20. The median is represented by the line within the box. The lines outside the boxes represent the maximum and minimum values. Outliers are denoted by hollow circles.

resistant strains of *O. tsutsugamushi* especially from the South Asian countries, there may be a need of an alternative treatment for combating ST burden [39–42].

Our study provides insights into the impact of IL-10 in ST infection in clinical cases and sub-clinical infections. Immunotherapy by blocking of IL-10 activity may be a good approach for treating scrub typhus infection, especially in endemic regions where Doxy- resistant strains of *O. tsutsugamushi* are prevalent. However, such approaches must be validated with studies aiming to identify the impact of monoclonal antibody induced blockade of IL-10 receptor. Limiting IL-10 can drastically enhance protective response that could have important implications in improving the vaccine design for this disease, as has been observed in some intracellular bacterial infections [43].

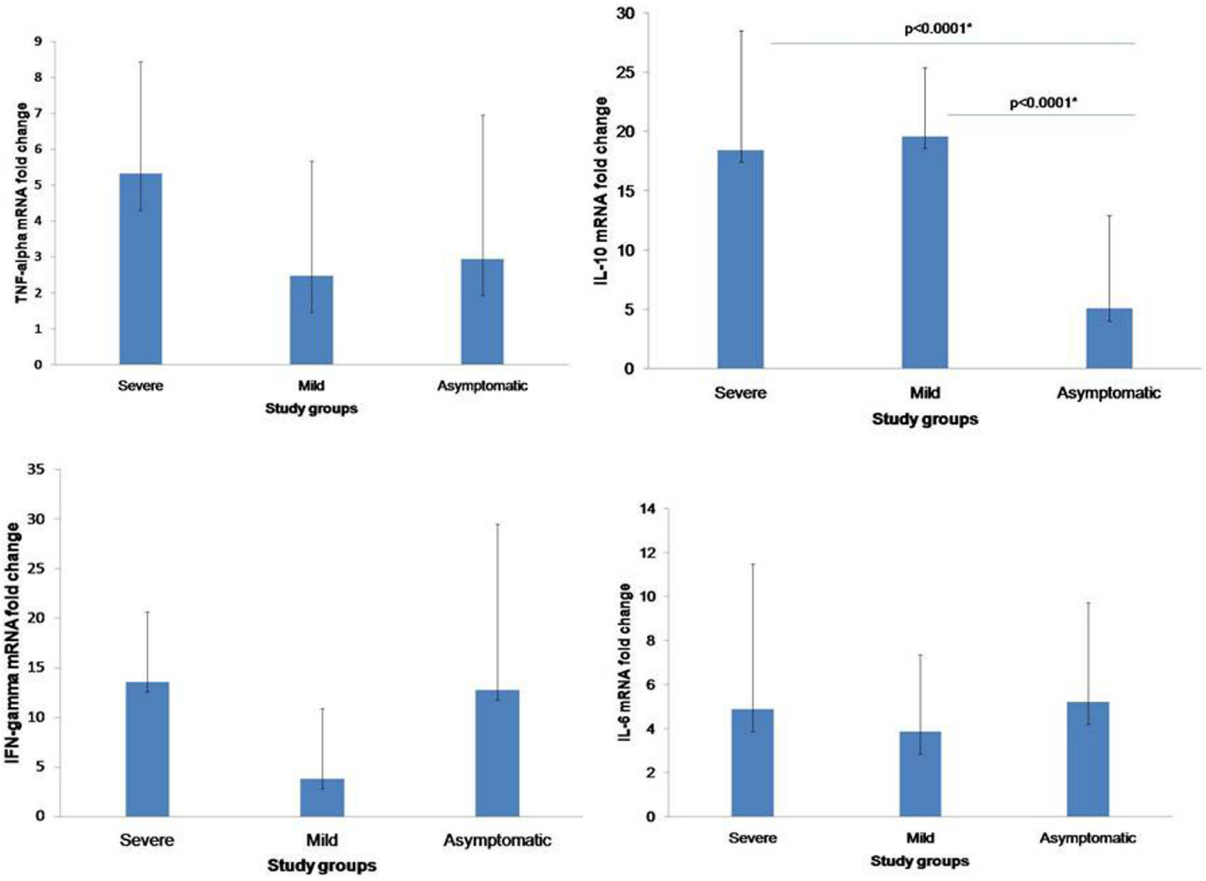

Fig. 3. Fold change difference in mRNA levels of the study cytokines by qPCR.

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Declaration of interest

None.

Ethical approval

Approval for the study was obtained from the Institutional Ethical Committee (IEC), RMRC, Dibrugarh, India vide letter no. RMRC/Dib./IEC (Human)/2014–15/860. IEC (human), RMRC, Dibrugarh follow the ethical guidelines developed by Indian Council of Medical Research (ICMR), New Delhi for biomedical research on human participants, 2006. The guidelines were developed in accordance with the Guidelines of Council for International Organizations of Medical Sciences (CIOMS) developed in 1964 at Helsinki and follow the Helsinki Declaration published in 2004.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.03.013.

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