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Multiple *Orientia* clusters and Th1-skewed chemokine profile: a cross-sectional study in patients with scrub typhus from Nepal

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ABSTRACT

Objectives: Scrub typhus is an emerging infectious disease in Asia caused by *Orientia tsutsugamushi* (*Ot*). From Nepal, only scant data on the genetic epidemiology of this agent is available, and determinants of immunoregulation are poorly understood.

Methods: Patients (n = 238) referred to the National Public Health Laboratory (Kathmandu, Nepal) from all over Nepal for suspected scrub typhus were enrolled upon positive immunoglobulin (Ig)M testing between July and October 2015. From *Ot* 16S and 47 kD polymerase chain reaction (PCR)-positive samples, the variable domain I of the 56 kD gene was sequenced and phylogenetically analyzed. T helper (Th) cell-associated cytokines (n = 13) and chemokines (n = 12) were quantified by multiplex bead arrays.

Results: In 93/238 (39.1%) IgM-positive samples, *Ot* DNA was detected by quantitative PCR. Phylogenetic analysis of 56 kD sequences revealed seven distinct clusters, six of them with high homologies to strains detected in other countries. The Th1-related cytokines interferon-γ and C-X-C motif chemokine ligand 10 were strongly upregulated and correlated with bacteremia, while levels of Th2-associated chemokines were reduced. Bacteremia also correlated with concentrations of interleukin (IL)-6 and IL-10 but not tumor necrosis factor-α.

Conclusion: We identified a considerable genetic heterogeneity of human-pathogenic *Ot* strains circulating in Nepal. Acute Nepalese scrub typhus patients showed strong Th1 but impaired Th2 responses, especially on the chemokine level.

Introduction

Scrub typhus, an acute febrile illness caused by the obligate intracellular bacterium *Orientia tsutsugamushi* (*Ot*), is a chigger-transmitted tropical disease and one of the leading causes of undifferentiated febrile illness in South Asia. Scrub typhus was historically reported to be endemic to northern and eastern Asia and a portion of northern Australia in an area referred to as the Tsutsugamushi triangle [1]. In recent years, an increased prevalence has been on the rise throughout the plains of South Asia, encompassing Nepal's [2] and Indian flatlands [3].

Until the devastating earthquake of April 25, 2015, scrub typhus had only been anecdotally reported from Nepal [4,5]. The steep increase in cases in its aftermath suggested that the impact of an earthquake facilitated exposure of the population to perturbed soil dwelling vectors [6]. Intensified clinical research thereafter showed that Nepal had a previously unrecognized burden of scrub typhus [2,7,8]. Despite these advances, a surprising gap of knowledge on *Ot* genetic epidemiology in Nepal and the infection-associated hu-

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man immune response has remained, and it was never studied whether a single strain may have caused the reported nationwide outbreak. Recently, one study reported *Ot* genotypes detected in patients from the Chitwan district, Nepal, which suggested the presence of one major dominant strain [8]. However, these rather local data cannot be regarded as representative of Nepal, which warrants further study of phylogenetic relationships of *Ot* strains in this country.

With its highly variable and immunodominant 56 kD surface protein, *Ot* has an appropriate gene target that is often used for phylogenetic analysis [9,10]. The 56 kD protein is also a vaccine candidate [11], which makes the collection of sequence information in emerging infection sites such as Nepal even more important.

Ot primarily infects tissue macrophages [12] and endothelial cells [13] in humans, but also other innate immune cells, including dendritic cells and monocytes [12], which was also demonstrated *in vitro* and in mouse models [14,15]. Upon infection with *Ot*, monocytic cells respond with the induction of cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10, and chemokines, e.g., IL-8, chemokine ligand 2 (CCL2) and C-X-C motif chemokine ligand (CXCL10) [16,17]. Increased levels of these cytokines in patients with scrub typhus [18] are therefore thought to reflect a strong innate immune activation. In addition, T cells contribute to cytokine production in human scrub typhus: Increased levels of granzymes and interferon (IFN)-γ levels in serum, and activated $CD8^+$ (cluster of differentiation 8) T cells with programmed death (PD)-1^{high} and IL-$7Ra^{low}$ phenotypes, are hallmarks of T cell activation during acute infection [19,20]. More specifically, IFN-γ is derived from antigen-specific $CD4^+$ Th1 cells and $CD8^+$ T cells that recognize peptides, e.g., derived from the outer membrane proteins 56 kD and ScaA [21]. In contrast, Th2 responses were found to be either absent or very weak in patients with scrub typhus [21,22], while one recent study in acutely ill travelers found some evidence for increased Th2-related cytokines (IL-4, IL-5, and IL-13) [23]. Thus, current models suggest a mixed cytokine profile of innate proinflammatory, IL-10, and Th1 immune responses in acute scrub typhus, but the relationship to bacterial concentrations has not been worked out.

A recent study demonstrated that monocyte-derived mediators, especially TNF-α, can activate the endothelium to express adhesion molecules and produce cytokines, while it, by itself, reacts poorly to *Ot* infection [24]. An excessive cytokine response is thus a critical factor in paving the way for endothelial pathology in scrub typhus. This is also reflected by the endothelial invasion of *Ot*, which is a hallmark of severe, fatal infection [13]. Indeed, increased levels of systemic cytokines (including IL-8 and TNF-α) have been found in severe infections and were associated with disease severity [18,25,26]. An interplay between cytokines and the endothelium is thus likely to influence the prognosis of *Ot* infection. Other severe complications in scrub typhus encompass hepatitis and renal failure, acute respiratory distress syndrome, myocarditis, and meningoencephalitis [27].

In addition, it is poorly understood how the concentration of circulating bacteria relate to the host's immune response. A better understanding of the complex immune responses elicited by *Ot* may help to develop new diagnostic algorithms that may better predict the disease prognosis and clinical complications.

The present study was conducted in a cohort of 238 acutely ill patients with scrub typhus from 47 Nepalese districts. We provide data on the detection and quantification of *Ot* DNA in serum samples and report partial 56 kD sequences from 25 patients. Moreover, with the use of multiplex cytokine and chemokine analysis, we demonstrate distinct profiles in patients with and without bacteremia.

Methods

Patient recruitment and controls

Suspected patients with scrub typhus with acute febrile illness who presented at the National Public Health Laboratory (Kathmandu, Nepal) were asked to enroll in the study between July and October 2015. A scrub typhus diagnosis was made upon clinical suspicion and serological confirmation by a positive Scrub Typhus *Detect*™ immunoglobulin (Ig) M enzyme-linked immunosorbent assay (ELISA) (InBios, Seattle, Washington, USA). Serum samples were collected during routine diagnostic investigation; 2 ml of serum was frozen and kept at -80°C for further analysis. A total of n = 238 individuals were enrolled. Patients originated from 47 different districts (Figure 1). Control subjects were healthy, asymptomatic individuals enrolled by the Kathmandu Research Institute for Biological Sciences (KRIBS), as described elsewhere [28]. Serum samples were stored at -80°C until further analysis. All participants provided informed consent.

Real-time quantitative polymerase chain reaction (qPCR) and conventional PCR

Nucleic acid was extracted from serum samples (0.2 ml) using the innuPREP Virus DNA/RNA Kit (Analytik Jena, Jena, Germany) on an automated Tecan extraction system (Tecan, Männedorf, Switzerland). Real-time qPCR using the *Ot*-specific 16S ribosomal RNA and 47 kD genes as targets were performed for molecular detection of *Ot* and quantification of bacterial DNA load [29,30]. For phylogenetic analysis, a fragment of the 56 kD gene (ca. 400 bp) was amplified by conventional PCR as described by Mahajan *et al.* [10] Details are available in supplementary methods. The 56 kD sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank (accession numbers ON236660-ON236684).

Quantification of cytokines and chemokines by multiplex bead array

Multiplex bead-based assays were performed for cytokine and chemokine profiling using LEGENDplex reagents (BioLegend, San Diego, California, USA), according to the manufacturer's instructions.

A pilot study was conducted in n = 30 acute samples (each with positive and negative PCRs) and n = 8 controls to analyze the entire set of 12 Th cytokines and 13 proinflammatory chemokines. Based on initial analyses, a subset of cytokines (IL-6, IL-10, IFN-γ, and TNF-α) and chemokines (Monocyte chemoattractant protein-1 [MCP-1] [CCL2], regulated upon activation, normal t cell expressed and presumably secreted [RANTES] [CCL5], inducible protein-10 [IP-10] [CXCL10], and IL-8 [CXCL8]) was selected for manufacturing of customized bead panels (BioLegend). This subset was measured in n = 64 samples and n = 50 controls. Protocol details are available in supplementary methods.

Statistical and phylogenetic analysis

Statistical analysis, including unpaired *t*-test, linear regression, and Tukey's multiple comparisons, was performed using GraphPad Prism version 8. For phylogenetical analysis, a ClustalW multiple alignment with sequences retrieved from the NCBI database was created in Geneious software (version 11.1.5). The neighbor-joining tree approach was used for construction of the phylogenetic tree using the Tamura–Nei model.

Results

The present study included n = 238 patients (median age 30 years [IQR 17-45]; sex ratio 51% male to 49% female, Table S1)

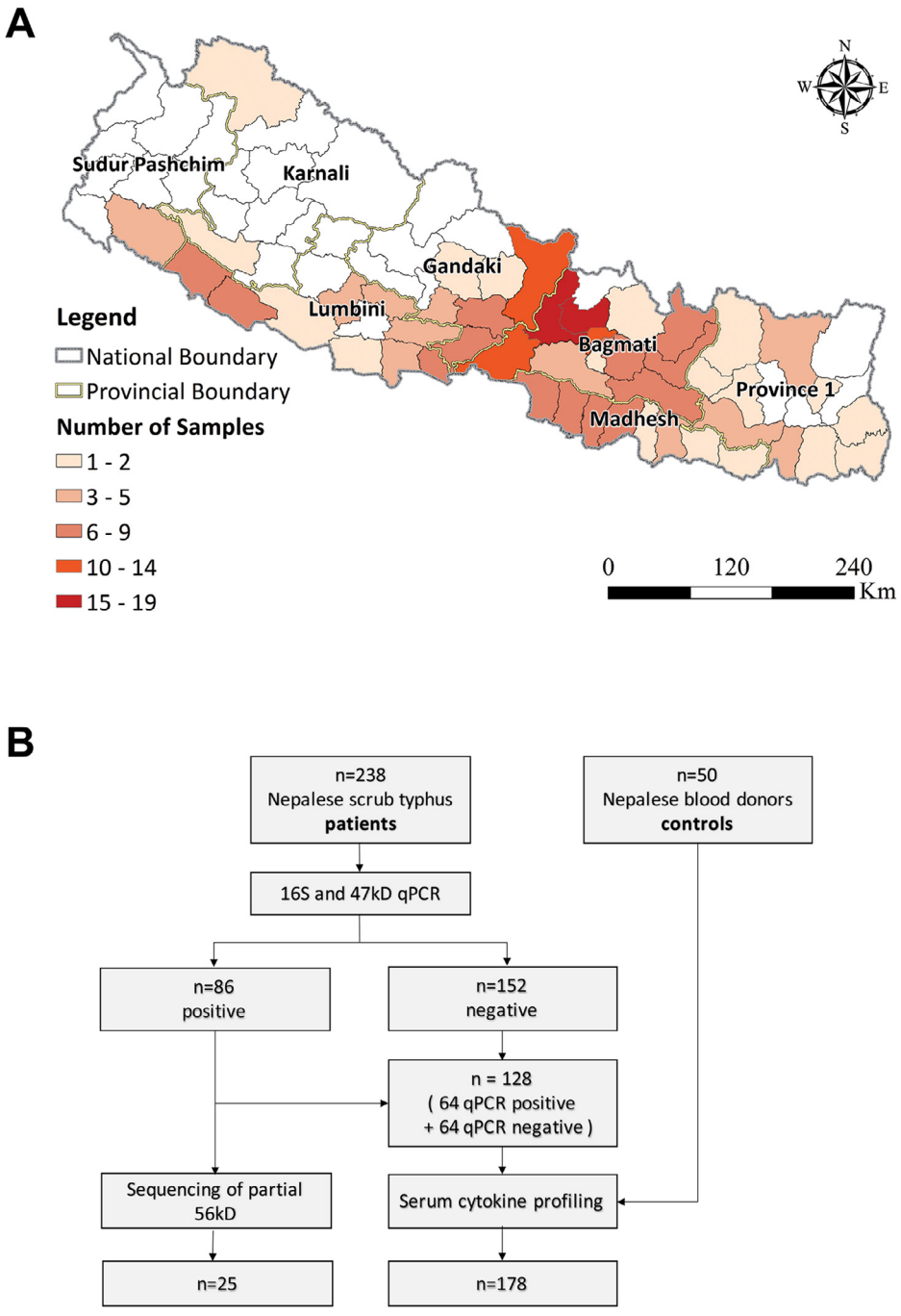

Figure 1. Reported residence of patients with scrub typhus and study design.
(a) Blood samples were collected from n = 238 *Orientia tsutsugamushi* immunoglobulin M enzyme-linked immunosorbent assay-positive patients with acute scrub typhus who reported residence in 47 districts in all seven provinces of Nepal. Districts with positive samples are marked in red, the intensity of the red color indicates the number of positive samples. (b) Serum samples from 238 patients were analyzed for bacterial DNA by 16S-and 47 kD-specific qPCRs. From qPCR-positive samples, a fragment of the 56 kD gene was amplified, sequenced, and phylogenetically analyzed. Serum levels of preselected cytokines were analyzed in 128 samples of scrub typhus patients (n = 64 with bacteremia, n = 64 without bacteremia) and compared to serum levels in healthy controls (n = 50).
qPCR, quantitative polymerase chain reaction.

who were clinically suspected of having acute scrub typhus and were tested positive by Scrub Typhus *Detect*™ IgM ELISA. Patients originated from 47 (of a total of 77) districts in seven provinces of Nepal, providing a broad geographical coverage for this study (Figure 1a).

High prevalence of Ot *DNA in serum samples*

Quantitative molecular testing for the *Ot*-specific 16S gene by qPCR from acute scrub typhus sera revealed a measurable bacterial load in 93/238 patients (39.1%, Figure 1b, Table S1), with a median of 410.3 16S gene copies/ml (IQR 55.31-2502). Bacteremia varied between 1 and 10^6 genome equivalents/ml (Figure S1a), resembling a normal distribution. Using a second qPCR targeting the 47 kD outer membrane protein gene, 86/93 16S-positive samples (92.5%) were confirmed (Figure 1b). A simple linear regression analysis revealed a close correlation between the 16S and 47 kD gene copies, with an R^2 of 0.4809 (P <0.0001, Figure S1b). This suggests that quantification results in this study were comparable by either gene target.

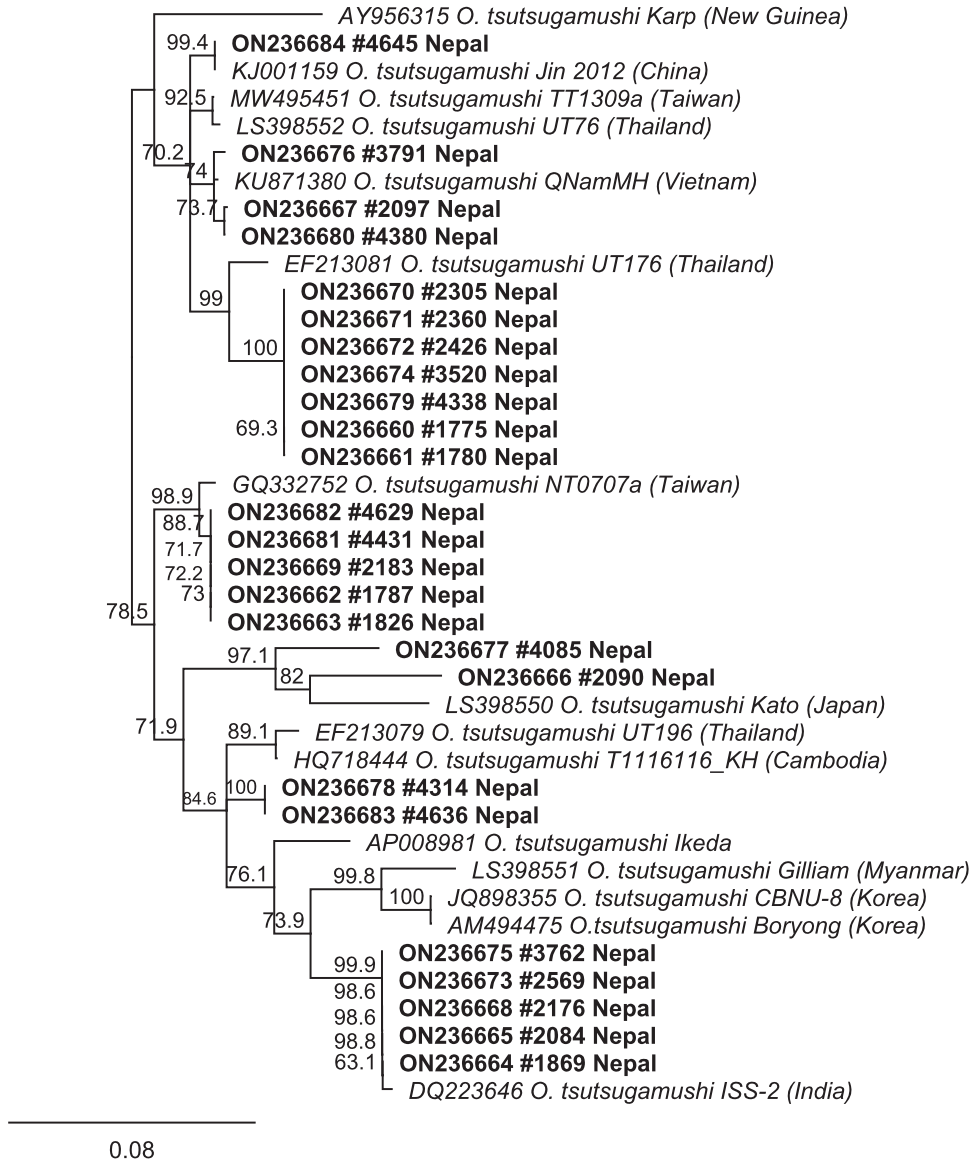

Figure 2. Phylogenetic tree of partial 56 kD sequences.
From n = 25 acute Nepalese scrub typhus sera, partial *Orientia tsutsugamushi* 56 kD sequences spanning the variable domain I were aligned by ClustalW multiple alignment, and a phylogenetic tree was constructed in Geneious 11.1.5 using the neighbor-joining tree method (Tamura–Nei model).

Phylogenetic analysis of partial Ot *56 kD sequences from Nepal*

To characterize the genetic diversity of human-pathogenic *Ot* strains from Nepal, partial sequences from the 56 kD gene, spanning the variable domain I (≈400 bases), were amplified and sequenced by Sanger sequencing. In 35/86 (41%) qPCR-confirmed samples, the 56 kD fragment was amplified. Sequences of sufficient quality were obtained in 25 of these samples (25/86, 29%). Samples with successful 56 kD amplification had a higher mean concentration of *Ot* in serum by 16S qPCR compared to those without (Figure S2).

As shown in Figure 2, the 25 Nepalese sequences clustered into seven different groups and showed high homology within each group: 7/25 (28%) were closely related to UT176, 5/25 (20%) to the NT0707a strain, 5/25 (20%) to ISS-2, 3/25 (12%) to the QNamMH strain, 2/25 (8%) to Kato, and 1/25 (4%) showed high homology with the Jin2012 strain. Of note, we did not identify one specifically Nepalese cluster of *Ot*. Instead, the sequences clustered most closely with strains detected, e.g., in Vietnam, India, Taiwan, Thailand, or China (Figure 2). Interestingly, 2/25 (8%) sequences, which were identical, displayed 10 mismatches with respect to the closest published strain and thus did not fall into any of the named prototype categories (sequences #4314; #4636). To our surprise, the sequences within a given cluster showed a high homology, despite their origins from distantly located sites, as depicted in Figure S3/Table S2.

Distinct cytokine/chemokine profiles in Nepalese patients with scrub typhus with and without bacteremia

To examine the relationship between bacteremia and serum cytokine levels, three groups were formed: patients with bacteremia (16S+), patients without bacteremia (16S-), and healthy blood donors as controls. For acute samples, patients were matched with respect to age, sex, and residence. First, a pilot study for analysis of 12 T helper cell subpopulation-associated cytokines and 13 chemokines (n = 30 patients per group, n = 8 controls) was conducted. Based on initial results, a selective analysis of four cy-

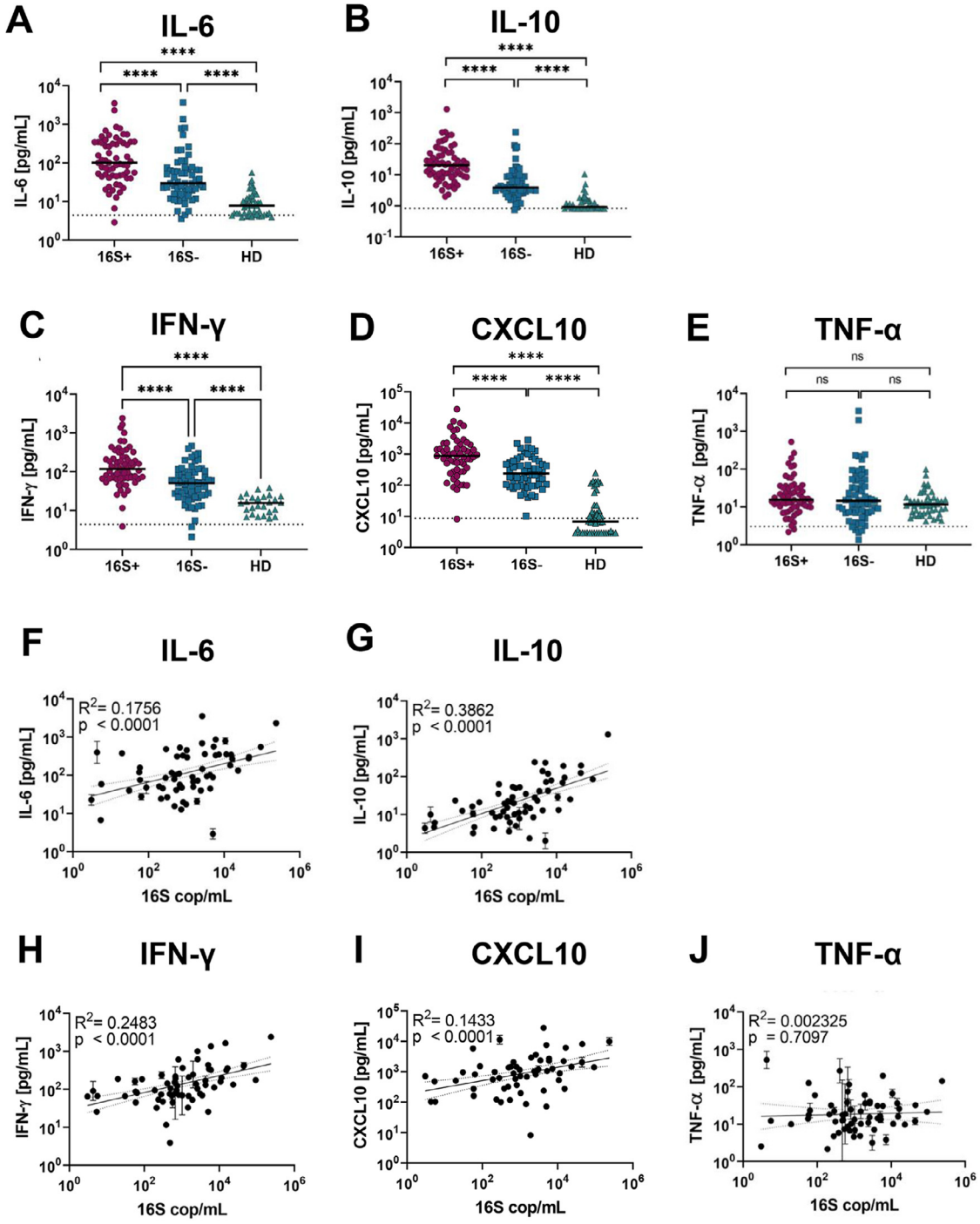

Figure 3. Plasma cytokine levels in patients with and without bacteremia.
The concentrations of the cytokines (a) IL-6, (b) IL-10, (c) IFN-γ, (d) CXCL10, and (e) TNF-α were measured in the serum of 16S qPCR-positive (16S+, n = 64) and -negative (16S-, n = 64) scrub typhus patients, and compared to HD (n = 50). Data are shown as median values. Dashed lines indicate the limit of detection. ****P <0.0001; ns by unpaired, two-tailed t-test. (f-j) Linear regression analysis between cytokine concentrations and bacteremia as measured by 16S qPCR was performed. Shown is the correlation of bacteremia with (f) IL-6, (g) IL-10, (h) IFN- γ, (i) CXCL10, and (j) TNF-α concentrations. Each dot represents one sample (mean ± SEM of technical duplicates). Best-fit line is shown together with 95% dashed confidence bands. Goodness of fit is indicated by R^2. The P-value indicates whether the slope is significant non-zero.
CXCL, C-X-C motif chemokine ligand; HD, healthy donors; IFN, interferon; IL, interleukin; ns, not significant; qPCR, quantitative polymerase chain reaction; TNF, tumor necrosis factor.

tokines (IL-6, IL-10, IFN-γ, and TNF-α) and four chemokines (MCP-1 [CCL2], RANTES [CCL5], IP-10 [CXCL10] and IL-8 [CXCL8]) followed; data were obtained for n = 64 patients per group and n = 50 controls.

As shown in Figure 3a-d, patients with scrub typhus, both with and without bacteremia, had higher serum levels of IL-6, IL-10, and the Th1-associated cytokines IFN-γ and CXCL10, compared to healthy donors (HDs) (P <0.0001). Also, patients with bacteremia had significantly higher levels of these four cytokines compared to patients without bacteremia (Figure 3a-d). In contrast to this, TNF-α levels surprisingly showed no difference between the three groups analyzed (Figure 3e).

Unlike in IFN-γ and CXCL10, we found no such upregulation for the Th2-associated cytokines IL-4, IL-5, and IL-13 or the other measured cytokines (Figure S4a-f). In fact, IL-5 levels were even lower in bacteremic patients compared to HDs (Figure S4c).

Hypothesizing that the presence of bacteria in serum could be a driver of increased cytokine responses, we investigated correlations between bacterial DNA and cytokine concentrations using a linear regression model. A positive correlation between bacteremia and IL-6, IL-10, and the Th1-associated cytokines IFN-γ and CXCL10 was found (Figure 3f-i). Surprisingly, concentrations of TNF-α ($R^2 = 0.002325$; P-value $= 0.7097$) were independent of bacteremia (Figure 3j).

To investigate whether the complex cytokine responses in bacteremic and non-bacteremic patients correlate internally, which would generate clustering of groups on a higher dimensional scale, the data set was visualized by t-stochastic neighbor embedding. Data sets for IL-6, IL-10, TNF-α, and the Th1-related cytokines IFN-γ and CXCL10 were included. The clustering pattern suggested distinct cytokine profiles in bacteremic and non-bacteremic scrub typhus patients, who were both clearly separated from HDs (Figure 4a). 2D heat maps demonstrated a positive correlation between IL-6 and IL-10 ($r = 0.68$), IL-6 and IFN-γ ($r = 0.67$), and between IL-6 and CXCL10 ($r = 0.41$) in bacteremic patients (Figure 4b, left panel). These correlations waned in non-bacteremic patients, where TNF-α now showed a positive correlation with IL-6 ($r = 0.60$), which differed from bacteremic patients ($r = 0.23$, Figure 4b, right panel). These differential correlations between IL-6 and IL-10, and IFN-γ and CXCL10 in bacteremic and non-bacteremic patients were confirmed by linear regression analysis (Figure S5). In contrast to the Th1 cytokines, the Th2-related cytokines IL-4, IL-5, and IL-2, showed no correlation to bacteremia (Figure S6a-c) or IL-6 (Figures 4a and b and S6d-f).

With regards to alterations of the chemokine signature, we also found that the mean concentration of IL-8 was elevated in scrub typhus patients, regardless of bacteremia status (Figure 5a). In the 13-chemokine panel obtained in the pilot subset, CCL2 and CXCL9 were only elevated in 16S-positive bacteremic patients (Figures 5b and c). Interestingly, in addition to IL-5, the chemokines CCL11 (Eotaxin), CCL17 (TARC), and CXCL5 (ENA-78) were also lower in scrub typhus patients compared to HDs, with and without bacteremia (Figures 5d-f). Other mediators were not significantly elevated or reduced in comparison to healthy controls (Figures 5g-l). The cytokine/chemokine signature thus involved elevated serum levels of IL-6, IL-10, IL-8, IFN-γ, and CXCL10 in patients with scrub typhus from Nepal, plus CCL2 and CXCL9 in those with bacteremia. Lower levels were found for CCL11, CCL17, and CXCL5 in patients with scrub typhus, plus lower levels of IL-5 in those with bacteremia.

We conclude that the cytokine/chemokine signatures differ between patients with scrub typhus with and without bacteremia, both in quality and quantity.

Discussion

This is the first comprehensive study on scrub typhus in Nepal that provides a deeper insight into molecular diagnostics, genetic epidemiology, and immunoregulation of human *Ot* infection in this country in a large cohort of $n = 238$ individuals.

First, we showed here that 39.1% of IgM-positive patients with scrub typhus had detectable *Ot* DNA in serum samples, which is within the reported range of PCR positivity in 25-65% of patients [31]. Due to the intracellular nature of *Ot*, an even higher DNA detection rate might have been obtained from cellular blood samples, such as buffy coat, as compared to serum samples [32]. Moreover, our study provides the first representative picture of the genetic heterogeneity of human-pathogenic *Ot* strains in Nepal, a topic previously addressed by only one study: Gautam *et al.* had concluded the existence of one main Nepalese cluster, which differed from strains detected in other countries [8]. With seven distinct clusters, we report a high genetic diversity of Nepalese human-pathogenic *Ot* strains. The different conclusion of the Gautam study may have been influenced by the study area that was restricted to one district (Chitwan) and a small number of samples ($n = 6$).

Interestingly, some newly found sequences showed high homology despite geographically distant origins. Studies from other countries, e.g., India and Laos, also reported a great in-country diversity and clustering of samples independent of geographical distance [33,34]. A reasonable explanation might be the association of certain *Ot* genotypes with specific mite vectors that are adapted to certain ecological habitats or the migration of strains by vectors or mechanical carriers, such as birds and rodents [35]. Also, the travel history of patients might play a role. One of the limitations of the present study is that a precise travel history was not obtained, so the acquisition of the sequenced *Ot* strains may not always have occurred at the residence indicated. Moreover, partial 56 kD sequence information, despite a frequently used tool for phylogenetic analyses [9,10], might not accurately mirror the actual genetic relationships, as would larger data sets from next-generation sequencing approaches [36]. However, 56 kD-based trees largely resemble trees that were constructed from the 657 core genes of *Ot* obtained by whole genome sequencing [37], suggesting that 56 kD sequences allow an approximation. The fact that 56 kD amplicons were obtained from only 25/86 qPCR-confirmed samples could be related to a much lower sensitivity of this assay, as suggested by previous reports [38]. In support of this interpretation, we found a lower mean bacteremia in samples without detectable 56 kD amplicon. The lower detection rate could also be related to suboptimal binding of 56 kD primers in some strains, so that the genetic diversity of *Ot* in Nepal might be even higher than found in this study.

The second part of the study focused on characterizing the cytokine profile in Nepalese patients with scrub typhus. We observed higher levels of IL-6, IL-10, and the Th1-associated cytokines IFN-γ and the chemokines CXCL10, CXCL9, IL-8, and CCL2 in our patient cohort. We also demonstrated a correlation of cytokine levels with the bacterial load, whereas patients without bacteremia showed lower levels of these cytokines. This suggests bacteremia is an important driver of these cytokines, e.g., via pattern recognition receptors (PRR). Although CXCL10 and CCL2 are typically involved in shaping the migration of Th1 cells [39,40], they likely represent an innate, possibly monocytic response to the pathogen [17]. IL-6 and the neutrophil attractant chemokine IL-8 are typically elevated innate cytokines in scrub typhus, as previously reported [18,23]. Upregulation of IL-10 in acute scrub typhus was also previously described [18] and shown to be associated with bacteremia [41]. IL-10 modulates the host response and inhibits Th1 immunity, which is thought to be essential for clearing *Ot*. It was postulated that *Ot* manipulates the host immune response by upregulating IL-10 to inhibit TNF-α production, thus restricting bacterial growth in macrophages [16,42]. By upregulating IL-10, *Ot* could be building a strategic environment for its survival and growth. TNF-α, on the other hand, showed no correlation with bacterial load in our study and also no difference to HDs, which is in contrast to observations in two studies from Thailand and in returning travelers [18,23]. Despite TNF-α being a marker of clinical severity [18,25,26], our data advocate that TNF-α may not be directly driven by bacteremia, and the elevated TNF-α levels found in severe cases of scrub typhus may be induced via PRR-unrelated mechanisms. Also, it is possible that our cohort comprised mainly mild scrub typhus cases; however, clinical data to confirm this were not collected.

High levels of CXCL9 and CXCL10 in bacteremic patients underline the prominent role of these cytokines in immune responses to

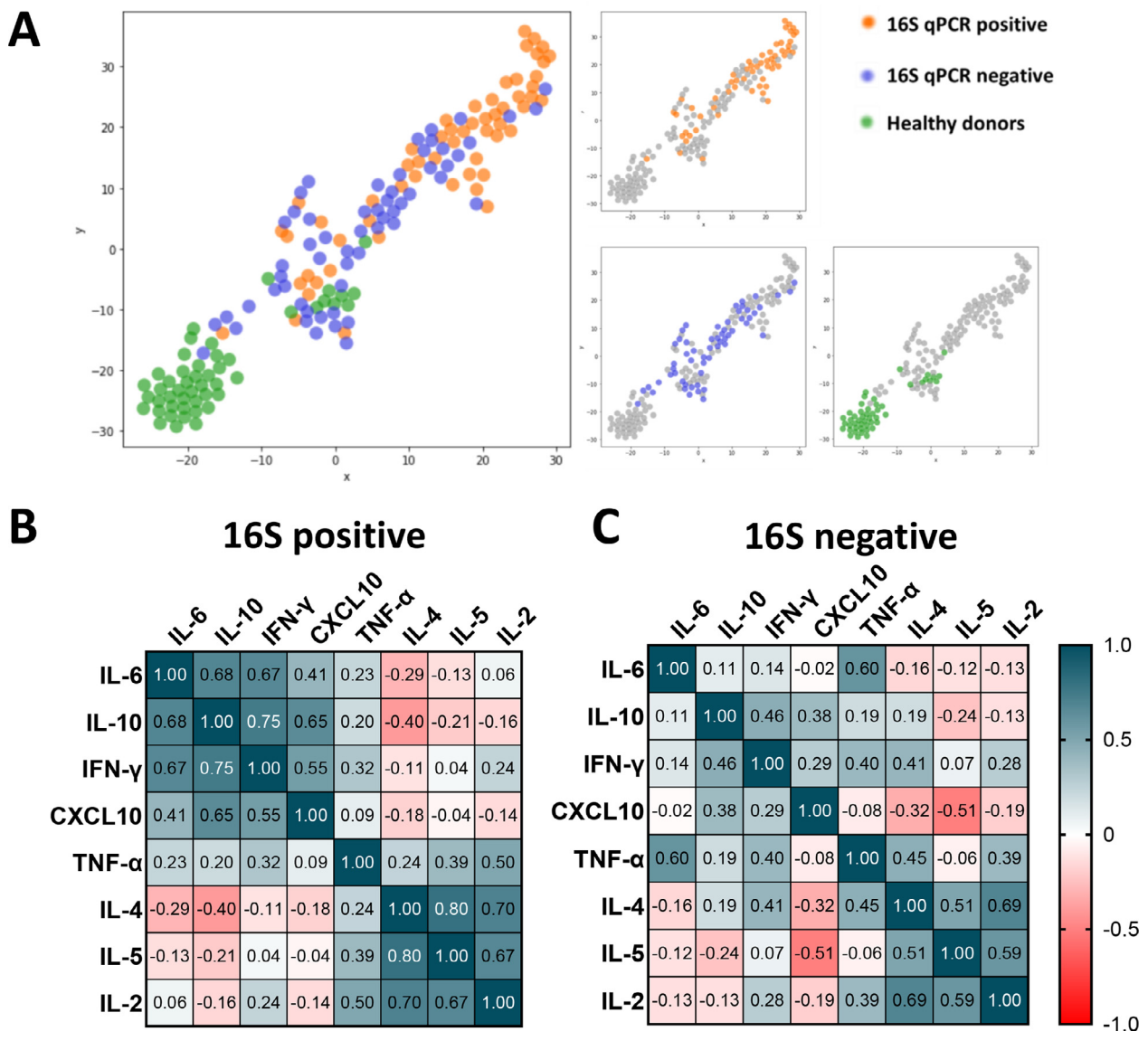

Figure 4. Distinct cytokine profiles in scrub typhus patients with and without bacteremia. (a) A t-distributed stochastic neighbor embedding plot was created from IL-6, IL-10, TNF-α, and CXCL10 concentrations, and bacteremia. Each dot represents one patient from the groups of 16S-positive (n = 64), 16S-negative patients (n = 64), and healthy donors (n = 50). (b, c) Heat maps with correlations between individual cytokines in 16S qPCR-positive (b) and 16S qPCR-negative scrub typhus patients (c). Pearson's correlation coefficient r is shown in each cell.
CXCL, C-X-C motif chemokine ligand; IFN, interferon; IL, interleukin; qPCR, quantitative polymerase chain reaction; TNF, tumor necrosis factor.

intracellular pathogens. CXCL10 (together with CXCL9) is the main ligand to the T cell chemokine receptor CXCR3, which is known to direct migratory behavior of T cells *in vivo*. This interaction is necessary to deliver effector T cells to specific anatomic locations, with CXCR3 driving T cells into lymphoid regions that are highest in antigen [43,44]. Moreover, CXCL10 affects the balance between effector and memory T cell generation [45]. The strength of inflammatory stimuli is crucial for the cell fate decision of effector vs memory T cells, with CXCL10-CXCR3 promoting commitment toward an effector phenotype [46]. Because *Ot* is an intracellular pathogen, it is interesting to note that CXCR3-mediated effects on memory T cell formation have been described in a model of mycobacterial infection before [47]. More research will be necessary to clarify how the plasticity of short-lived effector vs long-term memory cells can be targeted to improve the clinical outcome of *Ot* infections.

Of note, we also obtained data that support a skewed Th1/Th2 balance during acute scrub typhus. The Th2-associated cytokines IL-4 and IL-13 were not upregulated, and IL-5 levels were even lower than in healthy controls. On the chemokine level, concentrations of CCL11 (Eotaxin), CCL17 (TARC), and CXCL5 (ENA-78) were reduced, which was previously observed only for CCL17 [18]. These chemokines have been implicated in the development of Th2-associated conditions, e.g., gastrointestinal helminth infection [48] or allergic asthma [49]. CXCL5 is expressed by eosinophils, which are hallmarks of Th2 responses [50]. Th2 cells express a set of chemokine receptors that differ from Th1 cells, and interaction with their ligands dictates a different chemotactic behavior [40,51]. Thus, the lower serum concentrations of Th2-associated cytokines in patients with scrub typhus are consistent with impaired Th2 responses, especially during bacteremia, similar to observations in experimental mouse infections [52,53].

We found that only in bacteremic patients IL-6 responses correlated with other cytokines. The non-bacteremic patients could represent a later disease stage, in which IL-6 induction is sustained while other cytokines are already reduced, or IL-6 is regulated in-

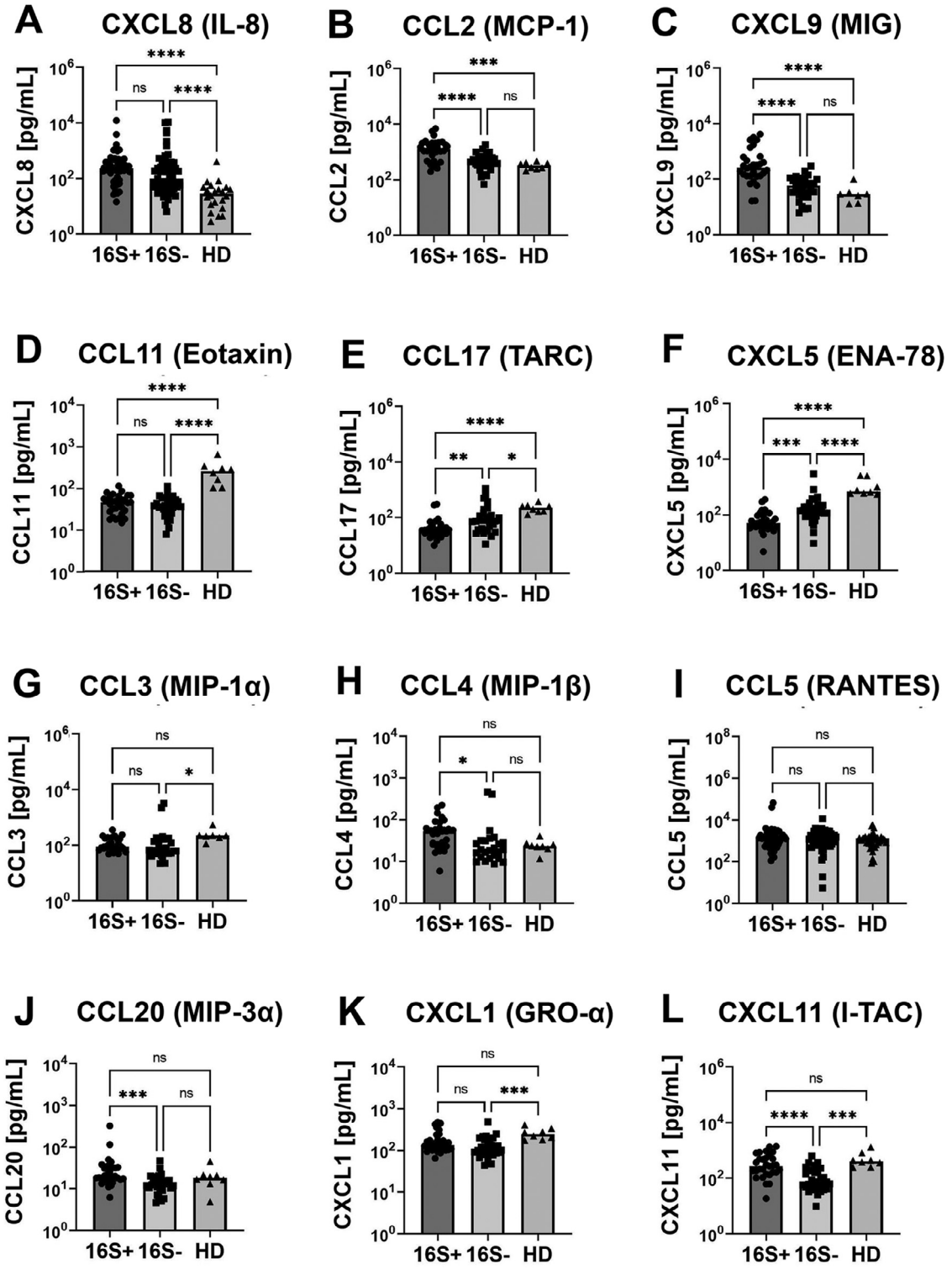

Figure 5. Th1-skewed chemokine profile in scrub typhus patients. (a-l) Serum levels of 12 chemokines (IL-8 [CXCL8], monocyte chemoattractant protein-1 [CCL2], monokine induced by gamma interferon [CXCL9], Eotaxin [CCL11], thymus and activation-regulated chemokine [CCL17], ENA-78 [CXCL5], MIP-1α [CCL3], MIP-1β [CCL4], regulated upon activation, normal t cell expressed and presumably secreted [CCL5], MIP-3α [CCL20], growth regulated-α [CXCL1], I-TAC [CXCL11]) measured by multiplex bead assay in scrub typhus patients with bacteremia (n = 30, 16S+), without bacteremia (n = 30, 16S-) and HDs (n = 8). Sample sizes for CXCL8 and CCL5 were n = 64 for patients and n = 50 for HDs. Data are shown as median values. $^{*}P$ <0.05; $^{**}P$ <0.01; $^{***}P$ <0.001;$^{****}P$ <0.0001, ns by unpaired, two-tailed *t*-test. Values below detection limit are not shown. CCL, chemokine ligand; CXCL, C-X-C motif chemokine ligand; HD, healthy donors; IL, interleukin; MIP, macrophage inflammatory protein; ns, not significant.

dependently in this phase. As another limitation of this study, we could not obtain follow-up samples upon convalescence to confirm this hypothesis.

In conclusion, our study reports the existence of at least seven human-pathogenic *Ot* clusters in Nepal. These clusters were closely related to *Ot* strains detected in other Asian countries. Moreover, the cytokine/chemokine profile in patients with acute scrub typhus was characterized by strong Th1 and impaired Th2 responses, and distinct cytokine/chemokine profiles were found in bacteremic and non-bacteremic patients. These data add to our understanding of scrub typhus pathogenesis and will be of help in the search for prognostic biomarkers.

Declaration of Competing Interest

The authors have no competing interests to declare.

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Ethical approval

The study protocol was approved by the Nepal Health Research Council (votes 22/2015, 172/2015, and 91/2017) and the Ethics Commission of Philipps University Marburg (vote 23/21).

Author contributions

CM: experimentation, data analysis and interpretation, manuscript draft. BPU: patient recruitment, study design. BR, AA: study design, graphics, manuscript draft. MM: data analysis. NE: experimentation. KK, ME: resources. CB: data interpretation, manuscript draft. KDM: study design. CK: study conceptualization, analysis, data interpretation, acquisition of funding, manuscript draft and revision. All authors reviewed the results and approved the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.12.022.

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