Specific CD4+ T-Cell Reactivity and Cytokine Release in Different Clinical Presentations of Leptospirosis

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Clinical manifestations of leptospirosis are highly variable: from asymptomatic to severe and potentially fatal. The outcome of the disease is usually determined in the immunological phase, beginning in the second week of symptoms. The underlying mechanisms, predictive factors, and individual immune responses that contribute to clinical variations are not well understood. The aim of this study was to determine the specifics of CD4+ T-cell reactivity and cytokine release after stimulation with leptospiral antigens in patients with leptospirosis of different disease severities (patients with mild and severe symptoms) and in control subjects (with and without proven exposure to Leptospira). Whole-blood specimens were stimulated with Leptospira antigens in vitro. Subsequently, intracellular staining of cytokines was performed, and flow cytometry was used to assess the expression of CD40 ligand (CD40L) and the production of gamma interferon (IFN-γ), interleukin-10 (IL-10), IL-2, and tumor necrosis factor alpha (TNF-α) by CD4+ T cells. The production of inflammatory cytokines such as TNF-α by CD4+ T cells after stimulation with leptospiral antigens was highest in patients with severe disease. In contrast, the ratio of IL-10 production to TNF-α production was higher in exposed subjects than in patients with mild and severe disease. Levels of proinflammatory cytokines such as TNF-α may be useful markers of the severity of the immunological phase of leptospirosis. IL-10 production by T cells after antigen-specific stimulation may indicate a more significant downregulation of the inflammatory response and may contribute to an asymptomatic course of the disease.

Leptospirosis is a zoonotic infectious disease caused by spirochetes of the genus Leptospira (1). The genus Leptospira includes 20 species and more than 300 serovars. Based on antigenic relatedness, 24 different serological variants have been identified in humans. In 2014, 160 cases of leptospirosis were reported in Germany (2), and the disease is considered to be a reemerging in temperate climates (3, 4).

The clinical presentation of leptospirosis varies widely from a mild, flu-like disease to severe, sometimes fatal infection (5). Clinically apparent disease is biphasic. The first phase is characterized by the presence of pathogens in the blood and may cause symptoms such as chills, fever, myalgia, rheumatic pains, and headache. This stage can last up to 1 week (6). The subsequent second immunological phase is characterized by the absence of the pathogen in the blood and the development of the immunological response with seroconversion (6). It may lead to a severe multiorgan failure (Morbus Weil, or Weil’s disease) (7). In recent years, life-threatening and fatal cases have been due most often to pulmonary hemorrhages caused by vasculitis of small vessels (1, 6, 8–10).

The mechanisms that influence the course of the disease are still not well understood, however. Some previous studies have investigated cytokine concentrations in serum samples in relation to the severity of the disease (11–14). Besides the general production of cytokines, CD3+ T cells play an important role in cell-mediated immunity against bacteria. While CD3+ CD8+ cytotoxic cells react to antigens produced within the respective cells presented by major histocompatibility complex (MHC) class I, CD3+ CD4+ T-helper cells interact with antigen-presenting cells that express MHC class II loaded with exogenous antigens. Thus, they are of special importance for the defense against extracellular bacteria such as leptospira. Following activation by recognized antigenic structures, CD4+ T cells activate B cells and produce cytokines. Depending on the costimulatory molecules of the antigen-presenting cells and the cytokine milieu, T-helper cells differentiate into distinct subpopulations that produce different cytokines. Inflammatory T-helper cells of type 1 (Th1) secrete predominantly interleukin 2 (IL-2), which promotes T-cell growth, and interferon γ (IFN-γ) and tumor necrosis factor alpha (TNF-α), which both activate macrophages to fight bacteria but also are known to mediate systemic inflammation. In contrast, IL-10 is an anti-inflammatory cytokine that is able to downregulate immune activation and to prevent immune hyperactivation. IL-10 originates mainly from CD25high FOXP3-positive regulatory T (Treg) cells and CD25high Treg-like T cells but to a lesser degree also from T-helper cells of type 2 (Th2). The role of the distribution of CD3+ T-cell populations, the specific CD4+ Th-cell reactivity to leptospiral antigens, and Th-cell-derived cytokines in association with disease severity has not been determined so far. Thus, the aim of this study was to characterize the specifics of CD4+ immune response and cytokine release in patients with...
mild and severe disease and in persons who had been exposed to *Leptospira* but did not develop symptoms.

**MATERIALS AND METHODS**

**Subjects and recruitment.** This was an observational study. We included patients with severe disease treated from 2003 to 2014 at the Department of Infectious Diseases of the Benjamin Franklin Campus of the Charité University Hospital in Berlin, Germany. Patients with less-severe disease were included from various other German hospitals. Subjects exposed to *Leptospira* but not showing symptoms were athletes who participated in a triathlon in 2006 that involved swimming through the Neckar River in Heidelberg. An outbreak of leptospirosis was reported during this triathlon (15). Age-matched, healthy controls were recruited as volunteers from the personnel of our hospital.

**Case definitions.** (i) Healthy controls were individuals without any known exposure to *Leptospira* spp. and without any symptoms or known risk of leptospirosis. Their test results for anti-*Leptospira* IgG and IgM as well as for leptospiral DNA assessed by PCR were negative. Additionally, none of the controls showed a leptospirosis-specific CD4+ T-cell response when we performed *Leptospira*-specific stimulation of their leukocytes, thus indicating that they had had no prior contact with pathogenic *Leptospira* spp.

(ii) Exposed subjects were triathlon athletes who had swum in the Neckar River in Heidelberg in 2006, during which time an outbreak of leptospirosis was reported. Their tests for IgG antibodies and IgM antibodies against *Leptospira* were negative, and the DNA of *Leptospira* spp. was not detectable (16–18). Testing was performed approximately 4 to 6 months after the triathlon. All of these subjects showed a leptospirosis-specific CD4+ T-cell response (characterized by the expression of CD40L) when we performed *Leptospira*-specific stimulation of their whole-blood samples, thus indicating that they had had prior contact with *Leptospira* spp.

(iii) Patients with mild symptoms had clinical signs such as flu-like symptoms, including headache, myalgia, polyarthralgia, fever, and chills. They were not treated at an intensive care unit. All patients in this group tested positively for anti-*Leptospira* IgG or IgM and/or had proof of the presence of leptospiral DNA. Testing for antibodies, leptospiral DNA, and CD4+ reactivity was performed approximately 2 to 4 weeks after infection. Thus, testing was conducted during the second phase of the disease, when antibody production takes place. All patients in this group had a positive history of exposure to *Leptospira* spp. Whenever possible, an additional test for CD4+ T-cell reactivity was performed after clinical recovery, 4 to 6 months after the initial infection.

(iv) Patients with severe symptoms and organ failure, such as pulmonary hemorrhages, were treated at intensive care units by therapy that included mechanical ventilation and administration of catecholamines. All patients in this group had seroconverted and had a positive PCR result. Testing for antibodies, leptospiral DNA, and CD4+ reactivity was performed approximately 2 to 4 weeks after infection. Thus, testing was performed in the second phase of the disease, when antibody production takes place. All patients in this group had a positive history of exposure to *Leptospira* spp. Whenever possible, an additional test for CD4+ T-cell reactivity was performed after clinical recovery, 4 to 6 months after the initial infection.

**Leptospira-specific leukocyte stimulation.** Antigen-specific effector CD4+ T cells were identified through short-term stimulation (19). Samples (500 μl) of fresh heparinized blood were taken from patients and exposed subjects 2 to 3 weeks as well as 4 to 6 months after infection or contact with *Leptospira*. These blood samples were then stimulated in 15-ml polypropylene tubes (Eppendorf, Hamburg, Germany) in the presence of αCD28 and αCD49d (20) (clones CD28.2 and 9F10; Becton, Dickinson and Company [BD], Heidelberg, Germany) (2 μg/ml; low endotoxin level; no NaN₃ and polymyxin B (Sigma-Aldrich, Taufkirchen, Germany) (1 μg/ml) for 6 h. Lysates of heat-inactivated *Leptospira* serovars Autumnalis, Bratislava, Copenhageni, and Pomona (each at 1 × 10⁷/ml) were used as antigens. These serovars were chosen because the patients revealed specific antigen production for at least one of these serovars. *Staphylococcus aureus* enterotoxin B (SEB) (Sigma) (2 μg/ml) served as the positive control, and the negative control contained only αCD28 and αCD49d. Brefeldin A (Sigma) (10 μg/ml) was added for the last 3 h of stimulation to assess cytokine production. At the end of incubation, 50 μl of 20 mM EDTA (pH 7.5) was added, and the reaction mixture was incubated for 10 min at room temperature and mixed vigorously. Nine volumes of fluorescence-activated cell sorter (FACS) lysing solution (BD) was added for 15 min at room temperature for lysis of erythrocytes and fixation. Specimens were washed and resuspended after fixation in phosphate-buffered saline (PBS)–0.5% bovine serum albumin (BSA)–0.02% NaN₃ (PBA) and stored at 4°C. The values from the negative control were subtracted from the values obtained after the stimulation. Any values measured after stimulation that were below the background level were defined as 0.

**Flow cytometric analysis.** Absolute cell numbers of CD3+CD8+, CD3+CD4+, and CD3+CD4+CD25high T cells were determined by the use of TruCount tubes (BD) and CD3/CD4/CD8 TriTest (BD), and mouse anti-human CD25 (clones 2A3 and M-A 251; BD) was used according to the manufacturer’s protocols. Samples from 95 healthy individuals were used as controls.

Antigen-specific CD4+ T cells were analyzed by four-color FACS analysis (19). Cell preparations were washed in PBA. The antibodies were then added, in 50 μl of PBA containing 2% BSA, 0.05% saponin (Sigma), for 15 min at room temperature for intracellular staining in dilutions determined before use (data not shown). Cells were washed and resuspended in PBA for analysis. Data were acquired on a FACScanLibur system (BD) and collected and analyzed with CellQuest (BD) and FlowJo (FlowJo LLC, Ashland, OR, USA) software.

Gates were set on lymphocytes on a side-scatter-plus-forward-scatter dot blot and on CD4+ cells. At least 50,000 CD4+ lymphocytes were analyzed. The following antibodies were used: anti-CD3 (SK7), anti-CD4 (SK3), anti-CD154 (CD40L, TRAP1), anti-IFN-γ (B27), anti-TNF-α (Mab11), anti-IL-2 (MQ1-17H12), and anti-IL-10 (JES3-19F1) (all from BD). Mouse IgG1, mouse IgG2b, and rat IgG2 (all BD) served as isotype controls. Levels of T-cell cytokines IL-2 and IFN-γ were measured in the context of CD4 and CD40L expression. Levels of the TNF-α and IL-10 that are also produced by monocytes and dendritic cells were measured without containing of CD40L but with the additional characterization of T cells by analysis of CD3 in addition to CD4.

**Statistical analysis.** The data are presented as single data points and means with 95% confidence intervals. The data were analyzed with the Kruskal-Wallis test. The Mann-Whitney U-test and paired t tests were used for post hoc analysis. We compared the values from the healthy controls with the values from both groups of patients and the values from the exposed subjects. The values from exposed subjects (which were all collected 4 to 6 months after exposure to *Leptospira*) were compared only to the patient values that were obtained 4 to 6 months after infection in order to ensure that the samples had equivalent time courses. P values of <0.05 were considered statistically significant and are noted in the figure legends.

**RESULTS**

**Subjects and clinical classification.** In total, we included 21 subjects (16 males and 5 females), with a mean age of 41.76 years (standard deviation, 11.45 years; range, 23 to 64 years). Their baseline and disease characteristics are presented in Tables 1, 2, and 3.

Healthy controls (n = 6) (Table 1, subjects 1 to 6) included two males and four females. The mean of their ages was 38.67 years (standard deviation, 13.46 years; range, 28 to 64 years).

Exposed subjects (n = 6) (Table 1, subjects 7 to 12) did not have any self-reported symptoms and included six males. The
mean of their ages was 36.17 years (standard deviation, 8.64 years; range, 23 to 48 years).

Patients with mild symptoms (\(n = 4\)) (Tables 2 and 3; subjects 13 to 16) had flu-like symptoms, including headache, myalgia, polyarthralgia, fever, chills, etc. (Table 3), and included three males and one female. The mean of their ages was 47.75 years (standard deviation, 11.84 years; range, 38 to 64 years).

Patients with severe symptoms (\(n = 5\)) (Tables 2 and 3, subjects 17 to 21) had high fever, meningitis, and organ failure such as pulmonary hemorrhages, kidney failure, or acute liver failure (Table 3). This group included five males. The mean of their ages was 46.2 years (standard deviation, 10.80 years; range, 38 to 59 years). Please note that one HIV-infected patient was included. The patient had already been treated with antiretroviral medication for 5 years, so that it was assumed that the patient has no deficient T-cell activity. Moreover, T-cells were counted at the time of the experiments, and the results (CD3, 824/\(\mu\)l; CD4, 250/\(\mu\)l; and CD8, 316/\(\mu\)l) showed that there was no immune deficiency.

### Phenotype of T cells
We compared the *in vivo* phenotypes of the T cells among the different groups before stimulation with leptospiral serovars. There was no difference either in absolute cell numbers of CD3\(^+\) (Fig. 1A), CD8\(^+\) (Fig. 1B), or CD4\(^+\) (Fig. 1C) T cells or in the percentages of CD8\(^+\) (Fig. 1D) and CD4\(^+\) among CD3\(^+\) T cells (Fig. 1E) for healthy controls, patients with mild disease, and patients with severe disease at 2 to 3 weeks and 4 to 6 months after infection and or for exposed subjects 4 to 6 months after the clinical exposure to *Leptospira*. But, in contrast to the results observed with healthy subjects, we observed an elevated percentage of CD25\(^{high}\) CD4\(^+\) T cells (which exhibit T-reg-like activity due to the production of IL-10) among the CD4\(^+\) T cells in patients with mild symptoms 4 to 6 months after infection and in severely affected patients 2 to 3 weeks and also 4 to 6 months after infection, as well as in exposed subjects 4 to 6 months after the exposure to *Leptospira* spp. (Fig. 1F).

#### Leptospira-specific CD4\(^+\) T-cell activation
Patients and exposed subjects revealed *Leptospira*-specific CD4\(^+\) T-cell reactivity as determined by the expression of CD40L (Fig. 2). In healthy controls, only the background level of CD40L\(^+\) among CD4\(^+\) T cells was detectable, and that level was significantly lower than the level seen with patients with mild disease 2 to 3 weeks after infection for stimulations with *Leptospira* serovars Autumnalis, Bratislava, and Copenhageni and 4 to 6 months after infection for stimulations with serovar Copenhageni. Comparing these results to those seen with severely affected patients tested 2 to 3 weeks after infection, the percentage of CD40L\(^+\) among CD4\(^+\) T cells was significantly reduced for all stimulations and 4 to 6 months after infection for stimulations with serovars Autumnalis and Copenhageni. In addition, healthy controls revealed a reduced proportion of CD40L\(^+\) CD4\(^+\) T cells compared to exposed subjects for stimulation with serovars Autumnalis and Copenhageni. Severely affected patients revealed an elevated proportion of CD40L\(^+\) among CD4\(^+\) T cells at 2 to 3 weeks compared to 4 to 6 months after infection for stimulations with serovars Autumnalis and Copenhageni.

### TABLE 1 Baseline characteristics of healthy subjects and asymptomatic subjects with or without proven exposure to *Leptospira*

| Subject | Sex | Yr of birth | Age (yrs) at analysis | Group | Exposure to *Leptospira* |
|---------|-----|-------------|-----------------------|-------|-------------------------|
| 1       | F   | 1967        | 40                    | Control | None                    |
| 2       | M   | 1963        | 44                    | Control | None                    |
| 3       | M   | 1942        | 64                    | Control | None                    |
| 4       | F   | 1981        | 33                    | Control | None                    |
| 5       | F   | 1986        | 28                    | Control | None                    |
| 6       | F   | 1985        | 29                    | Control | None                    |
| 7       | M   | 1977        | 30                    | Exposed | Triathlon in Neckar river |
| 8       | M   | 1967        | 40                    | Exposed | Triathlon in Neckar river |
| 9       | M   | 1969        | 38                    | Exposed | Triathlon in Neckar river |
| 10      | M   | 1984        | 23                    | Exposed | Triathlon in Neckar river |
| 11      | M   | 1969        | 38                    | Exposed | Triathlon in Neckar river |
| 12      | M   | 1959        | 48                    | Exposed | Triathlon in Neckar river |

* F, female; M, male.

### TABLE 2 Baseline characteristics of patients with leptospirosis, including patients with mild or severe disease symptoms

| Subject | Sex | Yr of birth | Age (yrs) at infection | Group | Necessity of intensive care unit | No. of days of hospitalization | Exposure to leptospira |
|---------|-----|-------------|------------------------|-------|---------------------------------|------------------------------|-----------------------|
| 13      | F   | 1949        | 64                     | Mild symptoms | No                             | 3                            | Gardening activity, including removal work on a pond |
| 14      | M   | 1969        | 38                     | Mild symptoms | No                             | 7                            | Triathlon in Neckar river |
| 15      | M   | 1958        | 49                     | Mild symptoms | No                             | 3                            | Triathlon in Neckar river |
| 16      | M   | 1967        | 40                     | Mild symptoms | No                             | 0                            | Swimming in free water in Brandenburg |
| 17      | M   | 1975        | 38                     | Severe symptoms | Yes (mechanic ventilation, katecholamines, dialysis) | 41                           | Trader for cotton wool and other fur |
| 18      | M   | 1957        | 57                     | Severe symptoms | Yes (mechanic ventilation, katecholamines, dialysis) | 18                           | Chef at an Asian restaurant |
| 19      | M   | 1955        | 59                     | Severe symptoms | Yes                             | 14                           | Swimming in Elbe River, regular gardening |
| 20      | M   | 1964        | 39                     | Severe symptoms | Yes (mechanic ventilation)      | 22                           | Gardening activity, including cleaning of a pond; contact with wild boars |
| 21      | M   | 1968        | 38                     | Severe symptoms | Yes                             | 18                           | Urine of infected domestic rats |

* F, female; M, male.
| Patient | Sex | Leading or main symptom(s) | Additional symptom(s) | Medication previous to hospitalization | Disease(s) previous to leptospirosis | Antibody status at time of analyses |
|---------|-----|----------------------------|-----------------------|---------------------------------------|-----------------------------------|-----------------------------------|
| 13      | F   | Polyarthralgia (knee, elbow, fingers, wrist joint) | Arthritis | Ibuprofen | Psoriasis and arthritis | IgM negative, IgG positive (against \(L.\) Bratislava [61 U/liter]) |
| 14      | M   | Flu-like symptoms | Flu-like symptoms | None | None | IgG positive (against \(L.\) Copenhageni [titer, 1:400] and \(L.\) icterohaemorrhagiae [titer, 1:800]) |
| 15      | M   | Polyarthralgia | Flu-like symptoms | None | None | IgG positive (against \(L.\) Copenhageni [titer, 1:100] and \(L.\) icterohaemorrhagiae [titer, 1:100]) |
| 16      | M   | Fatigue | Flu-like symptoms | None | None | IgG positive |
| 17      | M   | Acute kidney and liver failure | Fever, icterus, fatigue, polymyalgia, headache, pancytopenia | None (but regular cannabis use) | None | IgM positive, (initially) IgG negative, PCR positive |
| 18      | M   | Respiratory insufficiency | Fever, chills, icterus, thrombopenia, mild sedation | None Post-hepatitis B infection | IgM positive (against \(L.\) Autumnalis [titer, 1:200] and \(L.\) Copenhageni [titer, 1:6,400]), IgG positive (against \(L.\) Australis [titer, 1:400] and \(L.\) Bratislava [titer, 1:3,200]) |
| 19      | M   | Pancytopenia, petechiae, hemorrhage, bleeding | Fever, polyarthralgia, fatigue, headache, icterus, thrombopenia, acute renal failure | Ibuprofen | None | IgM positive, IgG positive, PCR positive |
| 20      | M   | Pulmonary hemorrhage, respiratory insufficiency | Fever, diarrhea, myalgia, SIRS, rhabdomyolysis, pancreatitis, myocarditis, preileus | None Hypothyreosis | IgG positive (against \(L.\) Pomona [titer, 1:1,280]) |
| 21      | M   | Fever of sudden onset, myalgia, arthralgia, and icterus | Canalicular cholestasis and ballooning of hepatocytes | Antiretroviral drugs HIV infection | IgG positive (against \(L.\) icterohaemorrhagiae [titer, 1:3,200], \(L.\) Copenhageni [titer, 1:200], \(L.\) Sejroe [titer, 1:100], and \(L.\) Pomona [titer, 1:100]), PCR positive |

| T-Cell Reactivity in Leptospirosis |

F, female; M, male; SIRS, systemic inflammatory response syndrome.
after infection for stimulation with serovars Autumnalis, Bratislava, and Pomona. Leptospira-specific CD4\(^+\) T-cell cytokine secretion. To characterize the Leptospira-specific T-cell reactivity in more detail, the percentage of CD4\(^+\) T cells producing cytokines upon stimulation with Leptospira spp. was evaluated in healthy controls, patients with mild symptoms, severely affected patients, and exposed subjects following Leptospira-specific stimulation as determined by flow cytometry are shown. Please note the different time points of analyses after infection. Values for 95 healthy control subjects were collected in our hospital in the course of routine diagnosis. Values corresponding to Mann-Whitney t test results are given in the figure. Please note that no data are shown for the distribution of T-cell subsets for patients with mild disease 2 to 3 weeks after infection. Patients either were not hospitalized or were discharged from the ward before the final diagnosis. In addition, two of the patients were diagnosed in Heidelberg, Germany, so fresh blood samples from those patients were sent to us by overnight courier service. Unfortunately, for the analysis used for the determination of T-cell subsets and CD25 expression, blood must collected at the day of analysis. Therefore, for these cases, only the antigen-specific stimulation procedure was performed. nd, not determined.

Leptospira-specific CD4\(^+\) T-cell cytokine secretion. To characterize the Leptospira-specific T-cell reactivity in more detail, the percentage of CD4\(^+\) T cells producing cytokines upon stimulation with Leptospira spp. was evaluated in healthy controls, in patients tested 2 to 3 weeks and 4 to 6 months after infection, and in exposed subjects tested 4 to 6 months after exposure (Fig. 3). The most prominent secretion was observed for TNF-\(\alpha\) at both 2 to 3 weeks and 4 to 6 months after infection. At the time of assessment when disease symptoms were present, the proportion of CD4\(^+\) T cells secreting TNF-\(\alpha\) was significantly elevated in patients with severe symptoms for all stimulations compared to controls (Fig. 3A). The percentage of TNF-\(\alpha\)-positive cells among CD4\(^+\) T cells was also significantly elevated in patients with mild symptoms 2 to 3 weeks after infection compared to controls for stimulations with serovars Autumnalis, Bratislava, and Copenhageni (Fig. 3A). After recovery from symptoms 4 to 6 months after the infection, the proportion of CD4\(^+\) T cells secreting TNF-\(\alpha\) was still significantly elevated in patients with severe symptoms compared to controls for stimulations with serovars Autumnalis, Bratislava, and Copenhageni (Fig. 3A) and compared to exposed subjects for all stimulations (Fig. 3A). Additionally, 4 to 6 months after infection, patients with mild symptoms revealed a significantly elevated production of TNF-\(\alpha\) compared to exposed subjects for stimulation with serovar Autumnalis (Fig. 3A).
IL-2 was another prominent cytokine in patients with severe symptoms of leptospirosis. Severely affected patients showed a significantly higher percentage of CD40L\(^+\) IL-2-positive cells among CD4\(^+\) T cells compared to healthy controls for all stimulations in the acute phase of infection. Patients with mild symptoms showed a significantly higher percentage of CD40L\(^+\) IL-2-positive cells among CD4\(^+\) T cells compared to healthy controls for stimulations with serovars Autumnalis and Bratislava (Fig. 3B). After 4 to 6 months, the proportion of CD40L\(^+\) IL-2-positive cells among CD4\(^+\) T cells was elevated in patients with mild symptoms compared to controls only for stimulations with serovars Autumnalis and Bratislava and in patients with severe symptoms only for stimulation with serovar Autumnalis (Fig. 3B).

Shortly after infection (2 to 3 weeks), the proportion of CD40L\(^+\) IFN-\(\gamma\)-positive cells among all CD4\(^+\) T cells was elevated in severely infected patients compared to healthy controls for stimulations with serovars Autumnalis and Bratislava, while patients with mild symptoms had a significantly elevated proportion of CD40L\(^+\) IFN-\(\gamma\)-positive cells among CD4\(^+\) T cells compared to controls for stimulations with serovars Autumnalis, Bratislava, and Copenhageni (Fig. 3C). At 4 to 6 months, the percentage of IFN-\(\gamma\)-positive CD40L\(^+\) CD4\(^+\) T cells was elevated in patients with severe symptoms compared to healthy controls and exposed subjects for all stimulations (Fig. 3C). Patients with mild symptoms had a significantly elevated percentage of IFN-\(\gamma\)-positive CD40L\(^+\) T cells compared to healthy controls for stimulation with serovars Autumnalis, Bratislava, and Copenhageni and compared to exposed subjects for stimulation with serovar Autumnalis 4 to 6 months after infection (Fig. 3C).

In contrast to inflammatory cytokines, the percentage of CD4\(^+\) T cells producing the anti-inflammatory cytokine IL-10 2 to 3 weeks after infection did not show any significant differences between healthy controls and patients with severe or mild symptoms (Fig. 3D). But 4 to 6 months after infection, the proportion of IL-10-positive cells among CD4\(^+\) T cells was elevated in exposed subjects, compared to healthy controls for stimulation with
In this study, we analyzed the *Leptospira*-specific CD4+ T-cell immune response and cytokine release associated with various severities of leptospirosis in order to identify the immunological factors that may predict the clinical severity of leptospirosis. We were able to demonstrate that exposure to *Leptospira* combined with an immunological recognition of *Leptospira* spp. does not necessarily result in clinical symptoms and specific antibody production. This was indicated by the specific T-cell reaction and cytokine signature seen in the group of exposed but asymptomatic subjects. We also showed that severely affected, mildly affected, and exposed asymptomatic subjects do have different cytokine patterns in retrospective analyses, which may be used as a marker for the severity of infection. The present data show that, overwhelmingly, inflammatory *Leptospira*-specific CD4+ T cells were found in subjects with severe life-threatening infections. Although the proportion of CD4+ CD25high T cells (which exhibit Treg-like activity due to the production of IL-10) among symptomatic patients rose after

![FIG 4](image_url)

**FIG 4** Production of cytokines by CD4+ T cells following *Leptospira*-specific stimulation in the course of infection. Data represent percentages (means and 95% CI) of cytokine-positive results among CD4+ T cells from severely affected patients 2 to 3 weeks and 4 to 6 months after the infection following *Leptospira*-specific stimulation as determined by flow cytometry. Percentages of TNF-α-positive results among CD4+ T cells (A) and percentages of CD40L+ IL-2-positive results among CD4+ T cells (B) are indicated. Note that data for TNF-α production were available for only 3 patients, and data for IL-2 production were available for only 4 patients. P values corresponding to paired t test results are given in the figure.

![FIG 5](image_url)

**FIG 5** Ratio of CD4+ T cells producing IL-10 versus TNF-α. Data represent ratios of the means of the percentages (individual values, means, and 95% CI) of IL-10-positive and TNF-positive results among all CD4+ T cells from patients with mild symptoms, recovered patients, severely affected patients, and exposed subjects following *Leptospira*-specific stimulation as determined by flow cytometry. P values corresponding to Mann-Whitney t test results are given in the figure.
infection, the T-cell regulation does not seem adequate to prevent inflammatory tissue damage.

One limitation of this study was the relatively small sample size for each group (n = 21 total subjects, 6 healthy controls, 6 exposed subjects, 4 patients with mild symptoms, and 5 patients with severe symptoms). Yet the most impressive cytokine production, which was significantly increased in patients with severe and life-threatening symptoms of *Leptospira* infection compared to exposed/ asymptomatic and healthy control subjects, was observed for TNF-α in our retrospective analyses. Interestingly, asymptomatic subjects with proven exposure to *Leptospira* showed no inflammatory CD4⁺ T-cell reactivity but instead showed an anti-inflammatory response characterized by a level of production of IL-10 higher than that seen with patients with severe or mild disease. Therefore, IL-10 might be protective and might prevent a severe course of infection or even the manifestation of symptoms. This finding is supported by previous non-*Leptospira* studies (21, 22). Furthermore, we also showed that the percentage of *Leptospira*-specific CD4⁺ T cells producing IL-2 and IFN-γ was higher in patients suffering from severe and mild symptoms than in exposed subjects and healthy controls.

Although efforts have been made, no valid or clinically relevant classification to predict severe leptospirosis has yet been developed (5). Comparisons to previous research are difficult, since only reports about cytokine concentrations in serum specimens have been published; no data have been published about human CD4⁺ T-cell cytokine release upon *Leptospira*-specific short-term stimulation in relation to the clinical severity of leptospirosis. One previous study did demonstrate, though, that naive human peripheral blood mononuclear cells (PBMC) in long-term culture can be stimulated by leptospiral antigens to produce IFN-γ, IL-12, and TNF-α (23, 24).

Some authors have proposed that severe cases of leptospirosis are differentiated from mild disease by a “cytokine storm” (14). Elevated cytokine production in severe disease compared to mild disease is consistent with our data, since patients with severe disease revealed more prominent production of all cytokines tested. It has been shown that extensive release of proinflammatory mediators such as TNF-α, IL-1β, and IL-6 causes pathological inflammatory disorders, tissue injury, and organ failure (25). Consistent with our findings, there seems to be a dominant role of TNF-α as a mediator of severe disease. High TNF-α levels have been linked with pulmonary hemorrhagic complications (13). In a study investigating the prevalence of leptospirosis in patients with acute hepatitis, an elevated TNF-α level was present only in a patient who died of leptospirosis (26). In line with those findings, proof of the presence of circulating TNF-α was associated with life-threatening kidney, liver, and lung involvement as well as hemorrhages and, thus, higher mortality, whereas TNF-α was not detected in patients without any of such organ dysfunctions (27).

While high TNF-α production seems to be associated with severe disease, the role of IL-10 is less clear. Elevated IL-10 levels have been shown to be associated with fatal cases (13) and to be higher in patients with severe disease than in those with mild disease (14). In addition, conflicting data about the predictive value of the IL-10/TNF-α ratio as a marker for disease severity in leptospirosis have been published. While two studies demonstrated that a high IL-10/TNF-α ratio was associated with lower disease severity (27, 28), one other study showed that a high IL-10/TNF-α ratio was linked with a severe or fatal outcome (13). The latter study result is in contrast to our data indicating that a large amount of proinflammatory TNF-α production may trigger severe clinical symptoms and courses of infection whereas a more balanced ratio of IL-10/TNF-α seemed to be predictive of a symptomatic infection. Thus, the anti-inflammatory T-cell activity demonstrated by the presence of CD25high Treg-like cells and the production of IL-10 in severely affected patients seems to be insufficient to prevent tissue damage induced by inflammatory cytokines.

Previous reports on IL-2 and IFN-γ are even more scarce. There has been only one relevant study on IL-2, which reported that it is elevated in severe disease (14). The only relevant data published on IFN-γ reported normal serum levels in Thai patients with leptospirosis (12). Contrastingly, findings may result from differences in group assignments, since previous studies lacked asymptomatic but exposed subjects. They also investigated the differences of cytokine concentrations in sera, whereas we analyzed CD4⁺ T-cell-associated cytokine production directly. Cytokines detected in the sera may be the product of many other cells activated by inflammation. Furthermore, cytokine production must be much more prominent to be detected by serum analysis than by direct intracellular analysis of cytokines produced by CD4⁺ T cells.

Consistent with the idea of an association between, first, an overwhelming inflammatory reaction and cytokine release and, second, disease severity, case reports of severe leptospirosis have been published which hint that glucocorticosteroid therapy may have a positive effect on the course of disease (29, 30). One of our patients among those severely ill with pulmonary hemorrhages was also successfully treated with glucocorticosteroids to ameliorate an extensive immune reaction. We therefore suggest discussing immune modulatory therapy with leptospirosis patients, at least those with severe symptoms, including organ failure.

Our report presents an association of *Leptospira*-specific inflammatory cytokine secretion of CD4⁺ T cells with a severe course of leptospirosis. Furthermore, IL-10 expressed by CD4⁺ T cells after contact with *Leptospira* seems to have a protective role, preventing (severe) symptomatic infection.

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