Clonal Accumulation of Activated CD8⁺ T Cells in the Central Nervous System during the Early Phase of Neuroborreliosis

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*Borrelia burgdorferi* may cause an acute infection of the central nervous system (CNS) that rarely leads to chronic disease. To characterize host immunity to *B. burgdorferi* in humans, we performed serial T cell receptor (TCR) variable β (TCRBV) chain analyses in blood and cerebrospinal fluid (CSF) samples from 10 patients with acute neuroborreliosis. In most patients, we found significant differences in TCRBV expression between CSF and peripheral blood T cells, predominantly involving CD8⁺ T cells. T cells that accumulated in the CSF had a memory phenotype and expressed high levels of C-C chemokine receptor 5 and CD69. Serial studies demonstrated that CD8⁺ T cell accumulation decreased continuously after resolution of the infection. In 2 patients, serial analysis of the TCR-α and -β chain sequences revealed that overexpression of TCRBV in CSF was caused by extensive clonal expansion of CD8⁺ T cells. Our findings support the role of CD8⁺ T cells during the early host defense against spirochete infection of the CNS.

The spirochete *Borrelia burgdorferi* is the causative agent of Lyme disease [1]. Lyme borreliosis can involve the skin, joints, heart, and central nervous system (CNS) [2] and is accompanied by a vigorous immune response against the spirochete [3]. The most common acute neurological manifestations are meningitis, cranial neuritis, and radiculitis [4, 5]. The syndromes are caused by bacterial infection of the meninges. Typical inflammatory changes in the cerebrospinal fluid (CSF) involve disruption of the blood-brain barrier and pleocytosis [4]. Neuroborreliosis is also characterized by a strong intrathecal antibody production against the spirochete [6]. After antibiotic treatment, most patients recover from acute disease, but, in some cases, a chronic inflammation of the brain or spinal cord develops in the absence of bacterial persistence [4]. Studies of Lyme arthritis and chronic neuroborreliosis suggest that spirochete-specific T cells cross-reacting with self antigens may be responsible for the autoimmune mechanisms leading to chronic disease [7, 8].

The immune response to *B. burgdorferi* has been studied extensively in infectious animal models [9, 10]. In susceptible rodents, infection with *B. burgdorferi* leads to a variety of organ manifestations, including myocarditis and arthritis. However, CNS infection is observed only in immunosuppressed monkeys [11, 12]. Pleocytosis is sometimes seen in these animals, but they do not show the symptoms of acute human neuroborreliosis. Although animal models have contributed...
significantly to the understanding of disease, the immune response in acute human neuroborreliosis has not been defined. Recent studies demonstrated cellular infiltrates of activated T and B cells in the CSF of patients with acute B. burgdorferi infection [13]. Furthermore, neopterine, interleukin (IL)–6, and matrix-metalloproteinases were detected at higher concentrations in the CSF of patients with neuroborreliosis than in the CSF of control subjects, which implies that a role exists for those molecules in the host response against infection [14–19]. Although B. burgdorferi–specific T cells seem to be present in the CNS of the patients, little is still known about the nature of the T cell response in patients with acute neuroborreliosis [20, 21]. Here, we investigated the T cell response in the CSF and blood at the onset of neuroborreliosis and during recovery.

PATIENTS, MATERIALS, AND METHODS

Patients. All patients were recruited at the Department of Neurology at Philipps-University (Marburg, Germany). Patients were enrolled only if they fulfilled at least 4 of the following 5 criteria: acute neurological symptoms consistent with radiculitis, cranial neuritis, or meningitis; mild-to-moderate paresis of a nerve root; meningitis; and during recovery.

Antibodies. The T cell receptor (TCR) variable β chain (TCRBV) antibody panel included TCR-BV1–phycoerythrin (PE) (clone BL37.2), BV2–PE (clone MPB2D5), BV3–fluorescein isothiocyanate (FITC) (clone CH92), BV5.1–FITC (clone Immunol57), BV5.2–FITC (clone 36213), BV5.3–PE (clone 3D11), TCRBV6.7 (clone OTI45), BV7–PE (clone ZOE), BV8–FITC (clone 56C5.2), BV9–PE (clone FIN9), BV11–FITC (clone C21), BV12–FITC (clone VER2.32.1), BV13.1–PE (clone Immunol22), BV13.6–PE (clone JU74.3), BV14–PE (clone CAS1.13), BV16–FITC (clone TAMAYA1.2), BV17–FITC (clone E17.5F3), BV18–PE (clone BA62.6), BV20–PE (clone ELL1.4), BV21.3–FITC (clone IG125), BV22–FITC (clone Immunol546), and BV23–PE (clone AF23) (all from Immunotech); CD4–allophycocyanin (APC) (clone RPA-T4; Becton Dickinson); and CD8–peridinin chlorophyll protein (PerCP) (clone SK1; Becton Dickinson). Iso-type-specific control antibodies (IgG-FITC, IgG-PE, IgG-PerCP, and IgG-APC; all from BD Pharmingen) were used to exclude the influence of nonspecific binding. Overall, the TCRBV panel covers ~70% of all known TCRs expressed on human T cells [22]. For additional characterization of CSF and blood T cells, antibodies against CD45RA (clone 0584; BD Pharmingen), CD45RO (clone UCHL-1; BD Pharmingen), CD28–APC (clone CD28.2; BD Pharmingen), CC15–FITC (clone 2D7; BD Pharmingen), CD31–PE (clone 1F11; Beckmann-Coulter), IL-7–PE (clone R34.34; Beckmann-Coulter), IL-12–PE (clone 114; BD Pharmingen), CD11a–FITC (clone LHI 111; BD Pharmingen), CD11b–FITC (clone BEAR1; Beckmann-Coulter), and CD8–PerCP were combined with the particular TCRBV antibodies.

Flow cytometry on whole blood and CSF cells. Fresh blood was diluted 1:1 with ice-cold PBS, and 200 μL of the mixture was added to each well of a round-bottom 96-well plate (Nunc). Plates were centrifuged at 200 g for 5 min, and the supernatant was discarded. The plates then were placed on ice, and cell pellets were resuspended by directly adding the monoclonal antibody. After 30 min of incubation on ice, erythrocytes were lysed in 2 steps, using 200 μL of ice-cold PBS containing 2% fetal calf serum (FCS), cells were transferred to 5-mL tubes (Falcon; BD Pharmingen) and analyzed on a 4-color flow cytometer (FACSCalibur; BD Pharmingen). For each stained blood sample, at least 10,000 events were acquired from a forward scatter–side scatter lymphocyte gate. Fresh CSF was spun down at 200 g for 10 min, the supernatant was removed, and the pellet was resuspended in PBS containing 5% FCS. Thirty microliters of the cell suspension was added to each well of a 96-well plate. Staining was performed as described above for whole-blood cells. No lysing step was applied to CSF cells, since no red blood cells were contained in these specimens. Cells were then washed twice and analyzed as described above.

Single-cell cloning, expansion, and RNA isolation. Freshly isolated CSF cells were spun down (as described above) and resuspended in Iscove’s modified Dulbecco’s medium (Gibco) containing 5% human serum, 1% l-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco) (T cell medium). CSF cells were diluted to 1 and 3 cells/well of a 96-well microtiter plate (Nunc) and mixed with 75,000 allogenic peripheral blood mononuclear cells (PBMC; 0.1 μL/well), stimulated with Dynabeads CD3/CD28 T cell expander (2.5 μg/mL; Dynal) or phytohemagglutinin (Sigma), according to the manufacturers’ instructions, and incubated at 37°C under 5% CO2. The cells were restimulated with human recombinant IL-2 (Diaclone) every 7 days starting at day 2 of culture. After 3 weeks of growing, T cell clones (TCCs) were screened for the expanded TCRBV chains using flow cytometry for CSF cells (as described above). Half the TCRBV-expressing (TCRBV+) cells from each well were further expanded by stimulation with CD3/CD28 beads, allogenic PBMC, and IL-2. The other cells were harvested for RNA isolation (RNeasy Mini kit; Qiagen). RNA was reverse transcribed to cDNA, according to the manufacturer’s protocol (Superscript; Gibco).
Polymerase chain reaction (PCR) and TCRBV sequencing. The cDNA was amplified using a set of primers specific for the TCRBV sequences, as described elsewhere [23]. After visualizing the products on agarose gel, products then were purified using a QIAquick PCR purification kit (Qiagen) and were sequenced using a DNA Sequencing kit (PE Biosystems) on an ABI Prism 310 Genetic Analyzer (PE Biosystems), according to the manufacturer’s instructions. All amplification steps were performed on a PTC-200 Thermal Cycler (Biozym). TCCs expressing identical or highly similar TCRBV complementary-determining region 3 (CDR3) regions were further analyzed for their TCRAV expression by use of the entire panel of TCRV-specific primers. The TCRAV chain then was sequenced as described above. The nomenclature used for the TCRBV-specific antibodies was that of Arden et al. [24], and the TCR-α and TCR-β sequences were analyzed according to the nomenclature of Rowen and Folch [25] and that of the international ImMunoGeneTics (IMGT) database [26] (available at http://imgt.cines.fr:8104/textes/IMGTrepertoire/). The single-letter code was used for amino acid sequences.

Quantitative reverse-transcriptase (RT) PCR. PBMC of patient 1 and PBMC of control donors (1 × 10⁸ cells each) were used to determine clonotype frequency by quantitative RT-PCR. To estimate the number of clonotypic T cells in the sample, we diluted different cell numbers of the TCC (10000, 3333, 1111, 370, and 123 cells) from patient 1 in 1 × 10⁸ PBMC of an unrelated donor. RNA was extracted from the different samples and used for the quantitative RT-PCR. We designed the following primers and probes for TCR genes by use of Primer Express 2.0 (Applied Biosystems): clonotype HJ1 TCR-β chain (forward, 5′-CCAGCCTCAGAAACCGG-3′; reverse, 5′-GGCTGTAGCCGGTCCGT-3′; and probe, 5′-ACTCAGCT-GTGACTTTCTGTGACAGCTTT-3′) and TCRB constant (C) region (forward, 5′-ACCCAGATCGTCAGCGCC-3′; reverse, 5′-AGACAGGACCCCTTGGGTG-3′; and probe, 5′-TAGAGCAC-GACTGTTGCTTTTACCTCGGTGTC-3′).

Taqman probes were labeled at the 5′ end with the reporter dye FAM and at the 3′ end with the quencher dye TAMRA (Eurogentec). Probes were phosphate blocked at the 3′ end. We performed the PCRs with a real-time RT-PCR kit (Eurogentec) and with 500 nM each primer, 150 nM probe, and 2.5 μL of diluted cDNA sample per reaction in a volume of 25 μL. The samples were assayed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Amplification conditions were 10 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C. We performed TaqMan RT-PCR for C-region and clonotypic amplification in duplicates. We adopted the comparative threshold cycle (Cₜ) method and used cDNA of a TCC expressing the clonotype sequence as the calibrator (equals 100%). First, the Cₜ for the clonotypic TCR (target) and the Cₜ for the C-region amplification (endogenous control) were determined for each sample. Differences in the Cₜ for the target and the endogenous control (ΔCₜ) were calculated to normalize for the differences in the RNA extractions and the efficiency of the RT step. The ΔCₜ for each experimental sample was subtracted from the ΔCₜ of the calibrator (ΔΔCₜ). The amount of clonotypic TCR cDNA in each sample was calculated by the formula 2⁻ΔΔCₜ. To estimate the number of clonotypic T cells in the blood sample, we compared the comparative concentration in this sample to the titration curve of the TCCs in unmatched PBMC.

Cytokine analysis. To determine the general cytokine secretion pattern of the TCCs, ∼50,000 cells of each TCC were seeded into each well of a 96-well round-bottom plate, washed once with T cell medium, and stimulated with 0.2 μL of CD3/CD28 beads in 200 μL of medium. After 24 h, supernatants were collected.

To analyze the response of the TCCs to B. burgdorferi antigens, we incubated dendritic cells (DCs) or Epstein-Barr virus–transformed B cells from patients 1 and 3 with different concentrations of B. burgdorferi lysates from cultured pBi, pKo, or JD1 strains, respectively. The cells were incubated overnight with the lysates, washed twice, and added to the TCCs. Controls were set up in parallel to measure the release of cytokines by DCs alone. After 48 h, the supernatants were collected, and the cytokine concentration was determined.

Cytokine analysis was performed by cytometric bead arrays (Th1/Th2 cytokine cytometric bead array; BD Pharmingen). In brief, 30 μL of supernatant was incubated in a well of a 96-well plate with 30 μL of cytokine detection fluid, consisting of 6 μL of cytokine bead mix (1 μL for each cytokine), 6 μL of detection reagent, and 18 μL of diluent buffer. At the same time, 30-μL standard samples containing 5–5000 pg/mL of each cytokine were incubated with 30 μL of cytokine detection fluid. After 3 h of incubation, 200 μL of wash buffer was added to the samples, and the plate was centrifuged. The supernatant was discarded, and the beads were resuspended in 150 μL and analyzed on the FacsCalibur. At least 1000 bead events were collected for each sample, and the mean value of PE staining was analyzed. The cytokine concentration of each sample was calculated on the basis of a standard curve.

Statistical analysis. Analysis of TCRBV chain staining was performed by normalizing proportions with respect to at least 2 different parameters. The expression of each given TCRBV segment (TCRBVn) was determined on cells that stained for a “main” phenotypic marker (e.g., CD4). The percentage of expression was calculated according to the following formula: (percentage of cells expressing both “main” and TCRBVn/percentage of all cells expressing “main”) × 100, where all cells expressing “main” include both TCRBVn-negative and -positive cells. To compare TCRBV expression between both compartments, the number of T cells that expressed a given TCRBV chain on CD4⁺
Table 1. Summary of clinical and laboratory findings.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, years</th>
<th>Sex</th>
<th>Symptom(s)</th>
<th>CSF Cell count, cells/μL</th>
<th>Protein concentration, mg/L</th>
<th>B. burgdorferi IgG index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>B. burgdorferi IgM index&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Class I type</th>
<th>Class II type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>M</td>
<td>Radicular low back pain</td>
<td>284</td>
<td>962</td>
<td>13</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A2, A7, B7, B39</td>
<td>DR15, DR1, DO0501, DO0602</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>M</td>
<td>Radicular low back pain, facial palsy</td>
<td>394</td>
<td>2206</td>
<td>1.1</td>
<td>0.2</td>
<td>A2, A24, B8, B44, C5, C7</td>
<td>DR3, DRA, DO02, DO03</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>M</td>
<td>Neck and shoulder-arm pain, paraesthesia in both hands</td>
<td>130</td>
<td>1119</td>
<td>3.3</td>
<td>1.5</td>
<td>A2, A2, B7, B18, C6</td>
<td>DR4, DR11</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>M</td>
<td>Radicular low back pain</td>
<td>333</td>
<td>2000</td>
<td>4.4</td>
<td>2.4</td>
<td>ND</td>
<td>DR4, DR13</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
<td>M</td>
<td>Radicular low back pain, paraesthesia in both legs</td>
<td>76</td>
<td>2200</td>
<td>0.6</td>
<td>3.2</td>
<td>A2, A3, B8, B57, C7</td>
<td>DR16, DR13, DO0501, DO0602</td>
</tr>
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<td>Neck and shoulder-arm pain</td>
<td>205</td>
<td>1660</td>
<td>2.6</td>
<td>2.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>69</td>
<td>M</td>
<td>Shoulder-arm pain, facial palsy</td>
<td>60</td>
<td>783</td>
<td>13.5</td>
<td>4.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>F</td>
<td>Paraesthesia in both legs and trunk</td>
<td>131</td>
<td>1860</td>
<td>2.3</td>
<td>0.9</td>
<td>A3, A30, B7, B51, C1, C7</td>
<td>DR15, DR1, DO05, DO06</td>
</tr>
<tr>
<td>9</td>
<td>72</td>
<td>M</td>
<td>Neck and shoulder-arm pain, facial palsy</td>
<td>61</td>
<td>680</td>
<td>10.7</td>
<td>3.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>74</td>
<td>M</td>
<td>Low back pain</td>
<td>171</td>
<td>2130</td>
<td>29.9</td>
<td>1.5</td>
<td>A25, A31, B35, B44, C4, C5</td>
<td>DR4, DR1, DO05, DO03</td>
</tr>
</tbody>
</table>

NOTE. CSF, cerebrospinal fluid; ND, not done.

<sup>a</sup> Defined as the highest *B. burgdorferi*-specific IgG antibody index observed during the course of disease: (B. burgdorferi IgG in CSF/B. burgdorferi IgG in serum)/(total IgG in CSF/total IgG in serum).

<sup>b</sup> Defined as the highest *B. burgdorferi*-specific IgM antibody index observed during the course of disease.

<sup>c</sup> IgM levels were below the detection limit.

or CD8<sup>+</sup> cells was determined in CSF and blood, and the relative percentages were subtracted; for example, for CD4<sup>+</sup> cells, the percentage of TCRBV<sub>n</sub> on CD4<sup>+</sup> blood was subtracted from the percentage of TCRBV<sub>n</sub> on CD4<sup>+</sup> CSF. Positive values indicate a relative enrichment of the given TCRBV in the CSF, whereas negative values indicate enrichment in peripheral blood. For statistical reasons, it is reasonable to assume that the staining for CD4 or CD8 and the different TCRBV chains are independent events. Therefore, we compared the number of TCRBV<sup>+</sup> and non–TCRBV-expressing (TCRBV<sup>−</sup>) CD4<sup>+</sup> T cells in both compartments by χ² analysis, to determine significant TCRBV expression differences. The application of the χ² test is appropriate because of the low number of CSF cells. The same method was applied to analysis of CD8<sup>+</sup> T cells. The χ² value for significance was 15 (<i>P</i> < .0001), because we used a global significance level of 5% in this study, together with a total number of 2 hypotheses, 10 patients, 22 stainings, and 2 different cell types. Bonferroni adjustments for multiple testing (22 TCRBV chains and 2 cell types) were done.

RESULTS

**TCRBV repertoire analysis demonstrates accumulation of CD8<sup>+</sup> T cells in CSF.** To investigate the T cell response in acute neuroborreliosis, we studied the TCRBV repertoire in the CSF and blood of patients at the time of diagnosis, before therapy was initiated. Ten patients with acute neuroborreliosis were enrolled in the study. Table 1 summarizes their clinical and laboratory findings. All patients had CSF pleocytosis, mainly consisting of T and B cells (data not shown). TCRBV expression was determined on CSF and blood T cells by 4-color flow cytometry, using a panel of TCRBV-specific antibodies that covers ∼70% of the human TCRBV repertoire. The use of antibodies allows precise determination of the number of TCRBV<sup>+</sup> cells and stratification of T cells according to other phenotype markers [22]. We combined the TCRBV-specific antibodies with markers for CD4 and CD8 molecules to establish the expression profiles on helper and cytotoxic T cells, respectively. We identified significant expression differences between CSF and blood T cells in acute neuroborreliosis. Likewise, in patient 3, only 1.9% of peripheral blood CD8<sup>+</sup> T cells but 10.1% of CSF CD8<sup>+</sup> T cells expressed the TCRBV5.1 chain (figure 1A). In contrast, TCRBV 20 was expressed at significantly higher levels on CD4<sup>+</sup> and CD8<sup>+</sup> T cells in blood, compared with T cells in CSF (figure 1B). All other TCRBV chains were found to be expressed at a similar level on CD8<sup>+</sup> and CD4<sup>+</sup> cells in the CSF and blood of this patient (figure 1B). These findings demonstrate a specific accumulation of CD8<sup>+</sup>
Clonal CD8⁺ T Cells in Neuroborreliosis

Figure 1.  T cell receptor variable β (TCRBV) chain expression on cerebrospinal fluid (CSF) and blood cells. A, Flow cytometric analysis of peripheral blood and CSF cells from patient 3 for the expression of CD8 and TCRBV5.1. Gating of lymphocytes in blood (left) and CSF (right), according to size and granularity, is shown on the upper panel; expression of CD8 and TCRBV5.1 is shown in the lower panel. Right, 10.1% of CD8⁺ CSF T cells express TCRBV5.1; left, 1.9% of blood CD8⁺ T cells express TCRBV5.1. B, TCR expression profile on CD4⁺ and CD8⁺ T cells from blood and CSF of patient 3. χ² values are displayed on the right. A χ² value >15 was considered to be significant, after adjusting for multiple testing. The staining for TCRBV6.7 could not be performed in this patient. C, Summary of TCRBV expression differences between T cells from CSF and peripheral blood for all patients studied. TCRBV chains that were expressed at significantly higher levels are displayed. The expression difference between blood and CSF is shown in parentheses. FSC, forward scatter; SSC, side scatter.

TCRBV5.1⁺ T cells in the CSF of this patient. Using stringent statistical criteria, we found similar TCRBV overexpression on CSF CD8⁺ T cells in 8 of 10 patients. In contrast, overexpression on CD4⁺ T cells was observed in only 1 of 10 patients (figure 1C). Accumulation of CD8⁺ T cells involved different TCRBV chains in individual patients, although overexpression of TCRBV5.1 was identified in 3 HLA-typed patients, 2 of them sharing the HLA-A2 allele. Of interest, in some patients, overexpression of particular TCRBV chains also was observed on peripheral blood CD8⁺ T cells, compared with the CSF repertoire, which suggests that these T cells are expanded in the periphery but do not infiltrate the CNS.

Phenotypic analysis of CD8⁺ T cells accumulating in CSF. Next, we investigated the phenotype of CD8⁺ T cells accumulated in the CNS. CSF and blood cells were stained with monoclonal antibodies specific for CD8, the overexpressed TCRBV chain, and antibodies specific for various molecules involved in T cell activation, maturation, and migration (see Patients, Materials, and Methods). TCRBV⁺ CD8⁺ CSF and blood T cells were gated and expression of those markers determined. The expression profile on TCRBV⁺ CD8⁺ T cells was compared with that of TCRBV⁺ CD8⁺ T cells and to TCRBV⁺ CD4⁺ T cells from CSF and blood. We found that CD8⁺ T cells that accumulate in the CSF of patients with acute neuroborreliosis are predominantly memory T cells (CD45RO⁺, CD45RA⁻, and CD28⁺; data not shown), as seen in other inflammatory CNS disorders [27]. The phenotype of CSF accumulated TCRBV⁺ CD8⁺ T cells, however, differed from the overall receptor expression of CD8⁺ TCRBV⁺ CSF T cells (figure 2). An up-regulation of the CD69 receptor and CCR5 was most evident on these cells, compared with other CD8⁺ CSF cells. Of interest, even in the peripheral blood, CD69 and CCR5
Figure 2. Phenotype analysis of CD8+ T cells accumulated in the cerebrospinal fluid (CSF). A, T cells from CSF and blood were stained with antibodies specific for CD8 and the expanded T cell receptor variable β (TCRBV) chain. These stainings were combined with antibodies specific for CD69 and CCR5. A representative example for the expression of CCR5 on TCRBV1-expressing (TCRBV1/H11001) and non–TCRBV1-expressing (TCRBV1/H11002) CD8+ T cells from CSF and blood of patient 4 is shown. B, Summary of phenotypic analyses for the expression of CD69 and CCR5 on CSF and blood T cells of 5 patients. Mean fluorescence intensity for each staining is shown. Patient 6 was used as control subject, because TCRBV5.1 was not overexpressed in the CSF of this patient. ND, not done.

were expressed at slightly higher levels on the TCRBV1+ CD8+ T cells, compared with those on the TCRBV- CD8+ T cells. In contrast, comparable expression differences were not observed for CD4+ T cells (data not shown).

Specific accumulation of CD8+ T cells decreases after microbial clearance. To understand the kinetics of the CD8+ T cell response, we performed serial analyses in several patients with neuroborreliosis before, during, and after antibiotic treatment. Some patients were monitored for up to 4 months for their TCRBV expression on CSF and blood T cells. In all patients, CSF white blood cell counts decreased rapidly during antibiotic treatment, reaching normal levels after ~4 months (figure 3). TCRBV chain expression analysis in all patients studied demonstrated that the overexpression of particular TCRBV chains was found at different time points during the course of disease, independently of the absolute white blood cell count (figure 3). TCRBV expression differences between CSF and peripheral blood CD8+ T cells were most prominent at the onset of disease and declined afterward. This phenomenon was most impressive in patients 3 and 4, in whom the accumulation of TCRBV5.1+ and TCRBV1+ CD8+ T cells reverted several months after the first spinal tap (figure 3). In contrast, overexpression of TCRBV20 in the peripheral blood of patient 3, which probably was not related to the spirochete infection, remained stable throughout the observation period, despite antibiotic therapy (figure 3).

Accumulation of T cells in the CSF is caused by clonotypic CD8+ T cells. Next, we investigated whether the accumulation of CD8+ T cells was caused by a clonal, oligoclonal, or polyclonal T cell population. To resolve this question, we cloned T cells of patient 1 from all 3 time points (figure 3). After 3 weeks, the cultures were screened for TCRBV8+ CD8+ T cells. TCCs expressing these markers were identified at each time point. RNA was extracted, reverse transcribed to cDNA, and amplified with TCRBV8-specific (or TCRBV12-specific, according to the IMGT database nomenclature [26]) primers. The amplificates were sequenced, and the CDR3 of the TCR-β chain was determined. At the first time point, 90% of all TCRBV8+ CD8+ TCCs expressed the same TCR-β chain, and
Figure 3. Serial T cell receptor (TCR) variable β (BV) chain profiling in patients with acute neuroborreliosis. TCRBV expression profiling at different time points is shown for 3 patients with acute neuroborreliosis. Nos. on the X-axes are cell counts. The white blood cell count in cerebrospinal fluid (CSF) is shown in the upper panel. Dashed arrow line in the upper graphs indicates the duration of the antibiotic treatment. A serial analysis of TCRBV expression for 22 TCRBV chains on CD8\(^{+}\) and CD4\(^{+}\) T cells is displayed in the middle and lower panel, respectively. The expression of each TCRBV chain was analyzed on CSF and blood cells, and the expression difference was determined. Positive nos. indicate overexpression on CSF, and negative nos. indicate overexpression on blood cells. The different symbols denote the different chains. TCRBV chains that were significantly overexpressed at different time points are highlighted by bold lines and the individual chain displayed in each graph. Dotted lines in the upper graphs indicate the treatment period.

47% of the TCCs did so at the second time point (figure 4A). Although only 2 TCCs were recovered from the last time point, both expressed the identical TCR-β chain sequence. In contrast, no sequence similarities of the CDR3 TCR-β sequences were found among 11 TCRBV8\(^{+}\) CD4\(^{+}\) TCCs established from the first time point (data not shown). The TCR-α chain sequences of the TCCs with identical TCR-β chain were then determined by a set of TCRA V-specific primers. All TCCs expressed the TCRA V14 chain, according to the IMGT nomenclature [26], with identical CDR3-α sequences (figure 4A). To determine whether this T cell was present in the peripheral blood of the patient, we established TCCs from the periphery. Of interest, the CSF clonotype was also highly prevalent in the peripheral blood, comprising up to 60% of TCRBV8\(^{+}\) CD8\(^{+}\) T cells. These findings formally prove that the overexpression of TCRBV chain on CD8\(^{+}\) T cells in the CSF is caused by an accumulation of T cells that originated from a single T cell. Given an absolute amount of 150 mL of CSF and a white blood cell count of 284 cells/µL, we estimate that >780,000 clonotypic CD8\(^{+}\) T cells were present in the CSF of the patient at the first time point. The absolute numbers decreased to ∼79,000 cells at the second time point and to 47,000 cells at the third time point. The observation that this T cell clone was also highly prevalent in peripheral blood demonstrates that the clone was expanded in the periphery but specifically accumulated in the CSF of this patient. Similar findings were obtained in patient 3, demonstrating that 62% of all CSF CD8\(^{+}\) T cells expressing TCRBV5.1 were of clonal origin (data not shown).

To ensure that the precursor frequency determined by limiting dilution cloning reflects the percentage of T cells in vivo, we performed a quantitative RT-PCR on PBMC of patient 1 from the second time point. Primers specific for the clonotypic gene and primers specific for the constant region of the TCR were used to quantify the frequency of the clonotype genes in comparison to all TCR-β chains. We determined the frequency of the clonotype in the peripheral blood on the basis of a standard curve (TCCs diluted in PBMC of an unrelated donor). We found that ∼2.8% of all CD8\(^{+}\) T cells in the blood expressed
Clonotype analysis of CD8\(^+\) T cells in cerebrospinal fluid (CSF) and blood. **A**, Serial analysis of T cell receptor (TCR)–\(\alpha\) and –\(\beta\) sequences of CD8\(^+\) TCR variable \(\beta\) (BV)–expressing T cells generated by limiting dilution assays from the CSF and blood of patient 1 (see figure 3, *left panel*). TCR–\(\alpha\) and –\(\beta\) complementary-determining region 3 regions of isolated clones are displayed. No. of T cell clones (TCCs) expressing the particular sequence and the percentage of the clonotype, compared with all TCCs generated from the particular time point, is shown on the right. The same clonotype (C1/B1) expressing TCRBV12/TCRAV14 (International ImMunoGeneTics database nomenclature [25]) was recovered from CSF and blood samples at all time points examined. AV, \(\alpha\) variable region; BJ, \(\beta\) junction region; ND, not done; NDN, hypervariable region. **B**, Analysis of clonotype frequency in blood of patient 1 (second time point) by real-time quantitative reverse-transcriptase polymerase chain reaction, using clonotypic and TCR–\(\beta\) constant region–specific primers. The observed clonotype frequency is compared to a standard curve established by diluting the TCCs into peripheral blood mononuclear cells (PBMC) from an unrelated donor (35% CD8\(^+\) T cells). The percentage of clonotypic T cells in relation to this particular TCR (figure 4B). This percentage is close to the expected 4.5%, as determined by cloning and flow cytometric analysis (7.5% TCRBV8* T cells, 60% clonotypic; see figure 1 and figure 3).

Cytokine profile of clonotypic T cells. The clonally expanded T cells were further investigated for their functional properties. In particular, we focused on the cytokine profile after in vitro activation by CD3/CD28 beads. Expanded T cells secreted large amounts of interferon (IFN)–\(\gamma\), little IL-2, less IL-4, IL-5, and tumor necrosis factor (TNF)–\(\alpha\), but no IL-10 after activation by CD3/CD28 beads (figure 5). The same cytokine profile was found to be expressed by clonotypic T cells established from different time points. In contrast, the cytokine profile from control CSF CD8\(^+\) T cell clones differed widely with respect to the secretion of the 6 cytokines (data not shown).

Next, we tested whether the TCCs responded to stimulation with lysate from in vitro–cultured spirochetes. Autologous DCs were generated and incubated with the *B. burgdorferi* lysate. The response of the TCCs after 48 h was measured by proliferation and cytokine assays. Preincubation of DCs with *B. burgdorferi* lysate induced a dose-dependent release of TNF–\(\alpha\) by the TCCs and a slight increase of IFN–\(\gamma\). DCs alone did not
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Figure 5. Cytokine profile of cerebrospinal fluid (CSF)–expanded CD8⁺ T cells. A, Cytokine secretion of CD8⁺ T cell clones (TCCs) generated from patient 1 from the first (1) and third (3) time point. B, Cytokine release of T cells after coculture with dendritic cells (DCs) that were preincubated with or without *Borrelia burgdorferi* lysate (left). The control experiment (DCs alone) is shown on the right. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

release TNF-α under this condition, demonstrating that the cytokines were produced exclusively by the T cells. We observed neither specific proliferation in this assay nor cytotoxicity of the TCCs when Epstein-Barr virus–transformed B cells pulsed with *B. burgdorferi* lysate were used as targets (figure 5).

**DISCUSSION**

The present study investigated the T cell response in the CSF of patients with acute neuroborreliosis, to obtain insights into possible mechanisms leading to chronic CNS disease. Our results support the role of CD8⁺ T cells in human acute neuroborreliosis by several lines of evidence. First, TCRBV repertoire differences between CSF and blood were most significant and more frequently observed on CD8⁺ T cells than CD4⁺ T cells. Second, the expression differences are caused by clonal accumulation of CD8⁺ T cells in the CSF. Third, CD8⁺ T cells that accumulate in the CSF display signs of in vivo activation by up-regulation of CD69 and CCR5. Fourth, accumulation of CD8⁺ T cells in the CSF vanishes after clearance of the microbe from the meninges. Finally, clonotypic CD8⁺ T cell clones from the CSF respond to *B. burgdorferi* antigens by the release of TNF-α.

The role of the T cell and antibody responses to spirochetes has been addressed in different experimental animal models and human Lyme disease. Humoral and cellular immune mechanisms are involved in the response to *B. burgdorferi* infection and are necessary to prevent chronic manifestation of disease [28, 29]. The inflammatory response specifically targets the spirochete, which is suggested by the correlation of inflammation with the presence of the spirochete in the affected tissue [30]. The immune response, however, is not only essential for clearing the infection but also may contribute to disease pathogenesis causing chronic inflammation [3]. Differential cytokine release by T helper cell subpopulations has been described as having a role in animal models. Th2 responses seem to be protective, whereas Th1 responses seem to be associated with the development of destructive disease [31, 32]. In chronic Lyme arthritis CD4⁺ T cells in the joint secrete predominantly Th1 cytokines [33, 34]. Similarly, *B. burgdorferi*–specific CD4⁺ T cell clones established from the blood of patients with neuroborreliosis secrete Th1 cytokines and, interestingly, IL-10 [20]. Previous reports of patients with Lyme arthritis also demonstrated that the TCR repertoire between joint and blood differs with an overexpression of particular TCRBV chains in the inflamed organ compartment [35, 36].
Less is known about the role of CD8+ T cells in Lyme disease. In patients with chronic Lyme arthritis, B. burgdorferi–specific CD8+ T cells were isolated from blood and joint fluid [37]. In mice, CD8+ T cells secreting large amounts of IFN-γ are observed after infection. Depletion of these cells decreases severity of arthritis [38]. Findings in the animal model suggest that B. burgdorferi antigens are able to enter the major histocompatibility complex (MHC) class I pathway in DCs [38], by either direct phagocytosis or uptake of liposome-like vesicles (so-called B. burgdorferi blebs) [39].

Although the target of CD8+ T cells accumulating in the CNS of patients with acute neuroborreliosis is as yet unknown, the temporal profile and the phenotype of the CD8 response support the idea that it is directly driven by the spirochete infection. Several possibilities can be discussed that may be responsible for the accumulation of CD8+ T cells. Most likely, these CD8+ T cells target spirochetal antigens presented on MHC class I. Among the possible mechanisms by which B. burgdorferi antigens enter MHC class I pathways in CNS cells, all of which express MHC class I at least in the activated state, are the direct uptake of spirochetal antigens via B. burgdorferi blebs or an intracellular persistence of B. burgdorferi in CNS cells [40–42], although the latter possibility is not yet confirmed. Alternatively, DCs, which are also found in the CSF of patients with neuroborreliosis, may capture the spirochete and present its antigens to the infiltrating CD8+ T cells [43, 44]. Since CD8+ T cells may not necessarily kill the expressing target cell but also promote other inflammatory responses, it is possible that the clonally expanded CD8+ T cells initiate the inflammatory response without destroying the presenting cells [45]. The release of Th1 and Th2 cytokines by the CD8+ clonotype recovered from the CSF in acute neuroborreliosis may promote not only cellular but also humoral immune responses in the CNS. Alternatively, TCCs may be activated by MHC-independent mechanisms related to the unspacific mitogenic properties and the costimulatory activity of spirochetal antigens. Experiments to determine B. burgdorferi antigen specificity in humans are impaired by the high variation of antigenic proteins expressed by the spirochete [46] and the host-specific antigen expression pattern of the spirochete [47]. Thus, proteins expressed by the spirochete in culture differ significantly from proteins expressed in the human host or even from the expression pattern in the nervous system. In the present study, we found evidence that the clonotypic TCCs isolated from the CSF release TNF-α after activation by the B. burgdorferi lysate, although we could not elicit any other T cell function. Because TCCs did not proliferate in response to the spirochetal antigen, it is most likely that the proteins that expressed B. burgdorferi cultures did not contain sufficient amounts of the antigen, which was initially responsible for the in vivo expansion of TCCs. However, the fact that the lysate induces TNF-α in the absence of any other T cell function may provide a possible mechanism for how the T cells mediate proinflammatory activity without directly harming CNS cells.

In summary, we have provided support for a possible role of CD8+ T cells during acute B. burgdorferi infection of the CNS. Ongoing studies will further address the role of CD8+ T cells in human neuroborreliosis. Characterizing the CD8+ T cell response may be important not only for future vaccination strategies against B. burgdorferi but also for understanding disease mechanisms in chronic Lyme disease.

References


