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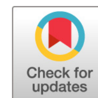

Peripheral T-Cell Reactivity to Heat Shock Protein 70 and Its Cofactor GrpE from *Tropheryma whipplei* Is Reduced in Patients with Classical Whipple's Disease

Lucia Trotta,[a,b]* Kathleen Weigt,[b] Katina Schinnerling,[b]* Anika Geelhaar-Karsch,[b] Gerrit Oelkers,[b] Federico Biagi,[a] Gino Roberto Corazza,[a] Kristina Allers,[b] Thomas Schneider,[b] Ulrike Erben,[b] Verena Moos[b]

First Department of Internal Medicine, University of Pavia, IRCCS Policlinico San Matteo, Pavia, Italy[a]; Medical Department for Gastroenterology, Infectious Diseases and Rheumatology, Charité-Universitätsmedizin Berlin, CBF, Berlin, Germany[b]

ABSTRACT Classical Whipple's disease (CWD) is characterized by the lack of specific Th1 response toward *Tropheryma whipplei* in genetically predisposed individuals. The cofactor GrpE of heat shock protein 70 (Hsp70) from *T. whipplei* was previously identified as a B-cell antigen. We tested the capacity of Hsp70 and GrpE to elicit specific proinflammatory T-cell responses. Peripheral mononuclear cells from CWD patients and healthy donors were stimulated with *T. whipplei* lysate or recombinant GrpE or Hsp70 before levels of CD40L, CD69, perforin, granzyme B, CD107a, and gamma interferon (IFN-γ) were determined in T cells by flow cytometry. Upon stimulation with total bacterial lysate or recombinant GrpE or Hsp70 of *T. whipplei*, the proportions of activated effector $CD4^+$ T cells, determined as $CD40L^+$ IFN-γ^+, were significantly lower in patients with CWD than in healthy controls; $CD8^+$ T cells of untreated CWD patients revealed an enhanced activation toward unspecific stimulation and *T. whipplei*-specific degranulation, although $CD69^+$ IFN-γ^+ $CD8^+$ T cells were reduced upon stimulation with *T. whipplei* lysate and recombinant *T. whipplei*-derived proteins. Hsp70 and its cofactor GrpE are immunogenic in healthy individuals, eliciting effective responses against *T. whipplei* to control bacterial spreading. The lack of specific T-cell responses against these *T. whipplei*-derived proteins may contribute to the pathogenesis of CWD.

KEYWORDS T-cell immunity, *Tropheryma whipplei*, Whipple's disease, cofactor GrpE, heat-shock protein 70

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Address correspondence to Verena Moos, verena.moos@charite.de.

* Present address: Lucia Trotta, Medicina Interna ad Indirizzo Pneumologico, Ospedale Fatebenefratelli ed Oftalmico, Milan, Italy; Katina Schinnerling, Programa Disciplinario de Inmunología, Facultad de Medicina, Universidad de Chile, Santiago de Chile, Chile.

L.T. and K.W. contributed equally to the publication. U.E. and V.M. contributed equally to the publication.

Classical Whipple's disease (CWD) is a rare chronic systemic infection caused by *Tropheryma whipplei*, a rod-shaped bacterium (1–4). The disease is diagnosed by periodic acid-Schiff staining of duodenal biopsy specimen, but early diagnosis remains difficult due to the strikingly heterogeneous clinical presentation. In the majority of patients, arthropathy precedes the involvement of other organs, such as the gastrointestinal tract, the nervous system, and the heart (5, 6). Since *T. whipplei* is common in the environment and genetically heterogeneous (7–9), the discrepancy among the widespread presence of *T. whipplei*, the frequency of self-limiting infections with *T. whipplei* (10), and the rarity of the disease is likely to be explained by immunogenetic host factors and susceptibility to the infection. It was recently shown that CWD is associated with the human leukocyte antigens DRB1*13 and DQB1*06 and that the cytokine genetic profile of CWD patients is skewed toward T-helper cell type 2 (Th2) and regulatory T-cell responses (11, 12). Family cases of the disease have also been described (13–15).

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Patients with CWD exhibit impaired *T. whipplei*-specific peripheral and mucosal Th1 responses (16) and a reduced serological reaction to *T. whipplei* proteins (17). In addition, alternatively activated macrophages predominating in the duodenal mucosa are unable to properly degrade the intracellular organism, leading to a persistent infection (18–21).

A serological proteomic approach identified candidate antigens of *T. whipplei* generating specific B-cell responses (22). Among these, GrpE, a cofactor of heat shock protein 70 (Hsp70) and a member of the highly conserved chaperone system necessary for protein folding, was identified to be involved in the interaction with the host immune system and to induce antibody production. As expected, expression of these proteins is highly upregulated in *T. whipplei* upon temperature stress (23).

Several facts demonstrate the role of immune reactions to heat shock proteins in protection against pathogens. Highly conserved Hsp70 itself is important in the defense against infectious agents, since early immune responses specific to heat shock proteins have been observed upon infections by bacteria, protozoa, fungi, or nematodes (24). An *in silico* analysis of known genes from *Leishmania infantum* uncovered potentially antigenic regions within the Hsp70 protein (25). Differential proteomics also established Hsp70 as a common antigen of *Helicobacter pylori* (26). Its adjuvant-type capacity even proved useful in tumor vaccination strategies, fusing Hsp70 to mesothelin or the oncoprotein E7 from human papillomavirus 16 (27, 28). In line with this, complexing potential antigens with a stress protein like Hsp70 or GroEL, belonging to the chaperonins in the chaperone family, provides potent immunogens for $CD8^+$ T-cell activation (29). In a mouse model, recombinant Hsp70 of *Salmonella typhi* alone established a predominant Th2 response subsequently protecting from lethal *Salmonella* infection (30).

We have previously shown that total lysate of *T. whipplei* Twist-Marseille activates peripheral and mucosal *T. whipplei*-specific Th1 cells of healthy individuals but does not elicit equivalent Th1 responses in CWD patients (16). However, the contribution of defined proteins from *T. whipplei* to the T-cell activation has not been defined so far. To evaluate the role of heat shock proteins in T-cell immunity against *T. whipplei* and to assess their potential diagnostic value to identify CWD patients, here we investigated the capacity of Hsp70 and of its cofactor, GrpE, to elicit specific proinflammatory $CD4^+$ and $CD8^+$ T-cell responses within the peripheral blood of CWD patients.

RESULTS

$CD4^+$ T-cell reactivity to recombinant *T. whipplei* proteins is reduced in CWD patients. To test the immunogenic potential of defined single *T. whipplei* proteins, the reactivity of $CD4^+$ T cells from whole blood samples of patients with different stages of classical CWD toward *Staphylococcus* enterotoxin B (SEB), *T. whipplei* lysate, heat shock protein 70 from *T. whipplei* Twist (TW-Hsp70), and GrpE from *T. whipplei* Twist (TW-GrpE) was compared with that of healthy donors. Hsp70 from *Escherichia coli* (EC-Hsp70) served as a control protein. As for the overall capacity of $CD4^+$ T cells to be activated, responses to SEB were reduced by half in untreated CWD patients compared to healthy individuals ($P = 0.014$) (Fig. 1A). Upon subdivision into patients with and without diarrhea, the differences in SEB reactivity were more prominent: untreated patients with diarrhea showed the lowest reactivity to SEB compared to control subjects ($P = 0.0004$); the reactivity was also reduced compared to untreated patients without diarrhea ($P = 0.023$) and reverted to normal upon treatment of CWD ($P = 0.014$) (Fig. 1A). The reactivity to SEB of untreated as well as treated patients without diarrhea was similar to that of control subjects (Fig. 1A). Central nervous system (CNS) infection did not significantly influence $CD4^+$ reactivity.

As against stimulation with SEB, specific $CD4^+$ T cell reactivity to *T. whipplei* lysate, Hsp70 and GrpE of *T. whipplei*, or EC-Hsp70 was not significantly influenced by treatment status of CWD patients ($P = 0.284$ as determined by Kruskal-Wallis test for multiple comparisons) (see Fig. S3A in the supplemental material). We also did not find any significant influence of disease manifestation on the reactivity to *T. whipplei* lysate

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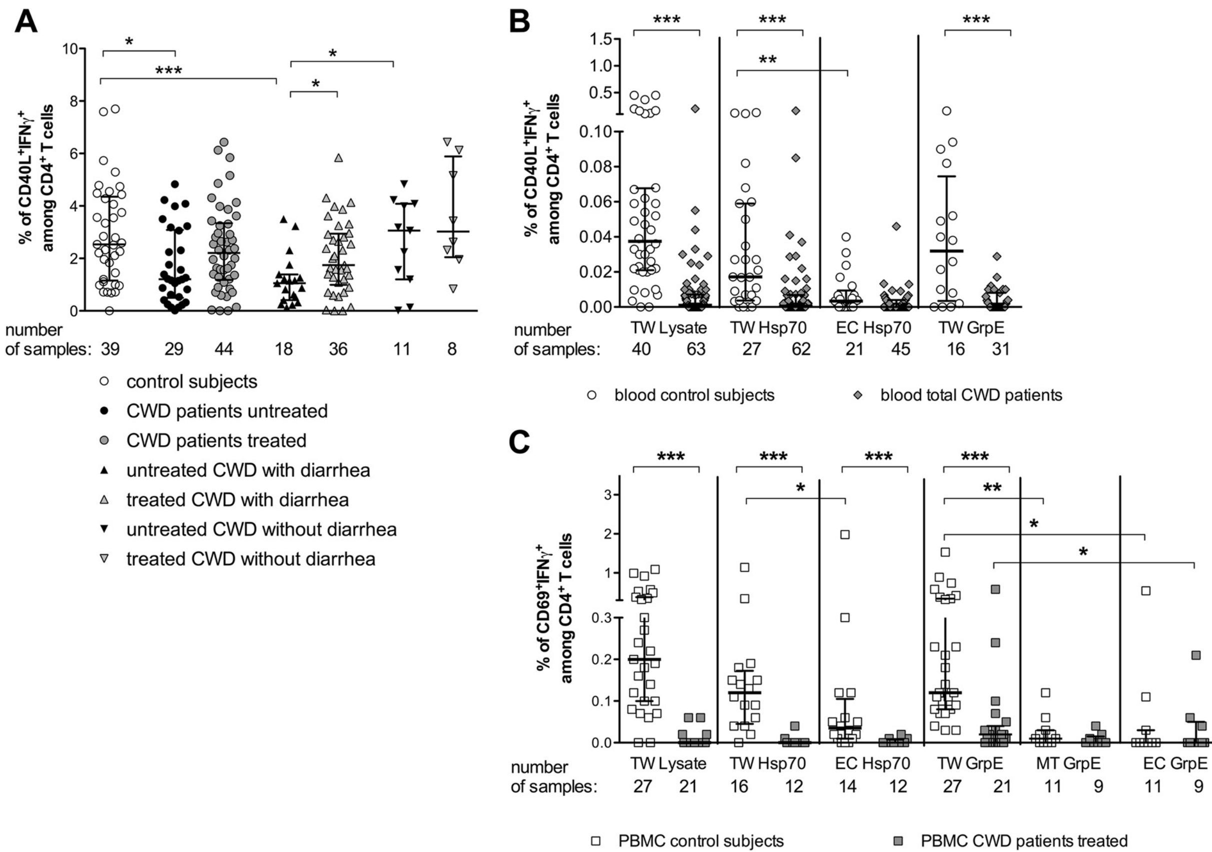

FIG 1 $CD4^+$ T-cell reactivity from fresh whole blood cells and cryopreserved PBMC. (A and B) SEB (2 μg/ml), *T. whipplei* lysate (10^7 bacteria/ml; TW lysate), recombinant Hsp70 from *T. whipplei* (80 μg/ml; TW-Hsp70), and *E. coli* (33 μg/ml; EC-Hsp70) or recombinant GrpE from *T. whipplei* (50 μg/ml; TW-GrpE) were applied for stimulation of whole blood of CWD patients and healthy controls. (C) TW lysate, TW-Hsp70, EC-Hsp70, and TW-GrpE, in concentrations given above plus recombinant GrpE from *M. tuberculosis* (50 μg/ml; MT-GrpE) and *E. coli* (50 μg/ml; EC-GrpE), were applied for stimulation of thawed PBMC of both CWD patients and healthy controls. Individual percentages and median values are shown for $CD40L^+$ IFN-γ^+ cells among $CD4^+$ T cells upon stimulation of whole blood with SEB (A), $CD40L^+$ IFN-γ^+ cells among $CD4^+$ T cells upon stimulation of whole blood (B), and $CD69^+$ IFN-γ^+ cells among $CD4^+$ T cells upon stimulation of PBMC as analyzed by flow cytometry (C). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ by Mann-Whitney *U* test.

($P = 0.826$ as determined by Kruskal-Wallis test) (Fig. S3A). In addition, the reactivity of $CD4^+$ T cells of CWD patients matched before and after treatment to *T. whipplei* lysate ($n = 11$), TW-Hsp70 ($n = 5$), and EC-Hsp70 ($n = 4$) did not reveal significant differences (see Fig. S3B in the supplemental material). Consequently, data of untreated and treated CWD patients were combined in one group for the assessment of specific reactivity. As described previously (16), CWD patients showed a significantly reduced percentage of $CD40L^+$ gamma interferon-positive (IFN-γ^+) cells within the $CD4^+$ T-cell population upon stimulation with *T. whipplei* lysate compared to controls ($P < 0.0001$) (Fig. 1B). Most interestingly, the reactivity to Hsp70 ($P < 0.0001$) and GrpE of *T. whipplei* ($P = 0.0003$) was significantly lower in $CD4^+$ T cells from CWD patients than in those from healthy donors. The percentage of $CD4^+$ T cells responding to EC-Hsp70 was generally very low and did not differ between control subjects and CWD patients (Fig. 1B).

Since fresh whole blood is a fragile material that cannot be stored, we aimed to address whether specific T-cell reactivity toward recombinant *T. whipplei* proteins can be tested from cryopreserved cells. Thus, we stimulated freshly thawed peripheral blood mononuclear cells (PBMC) analogous to whole blood with Hsp70 and GrpE of *T. whipplei* and Hsp70 of *E. coli*. As additional control proteins, GrpE from *M. tuberculosis* (MT-GrpE) and *E. coli* (EC-GrpE), representing pathogenic and commensal bacteria, respectively, were recombinantly expressed and included into these stimulations (Fig. 1C). Since CD40L is a reliable marker only for early T-cell activation (31) and cryopre-

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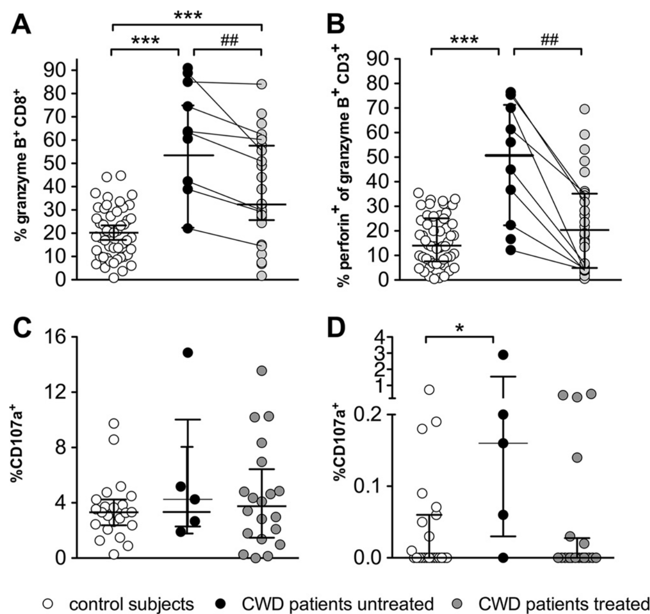

FIG 2 *In situ* activation state of peripheral CD8$^+$ T cells. Fresh whole-blood samples stained for CD8, granzyme B, perforin, and CD107a expression in fresh whole blood stimulated with SEB and *T. whipplei* lysate were analyzed by flow cytometry. Individual percentages and median values of granzyme B$^+$ cells among CD8$^+$ CD3$^+$ T cells (A), perforin$^+$ granzyme B$^+$ cells among CD8$^+$ CD3$^+$ T cells (B), and CD107a$^+$ cells among CD8$^+$ CD3$^+$ T cells (C) following stimulation with SEB of *T. whipplei* lysate (D) in untreated and treated CWD patients compared with healthy controls are shown. ***, *P* value of $<$0.001 by Mann-Whitney *U* test; ##, *P* value of $<$0.01 for paired data of untreated versus treated patients by Wilcoxon signed-rank test.

served cells required incubation for 12 h, we assessed activation by measurement of CD69 expression. However, the comparison of the percentages of CD40L$^+$ IFN-γ^+ CD4$^+$ T cells of matched fresh whole blood and thawed PBMC of each of five CWD patients and control subjects produced similar results. As an example, the stimulation with TW-Hsp70 and SEB is illustrated in Fig. S4. Comparable to the cells from fresh whole blood, the proportion of activated CD4$^+$ T cells reacting specifically to *T. whipplei* lysate or to Hsp70 or GrpE of *T. whipplei* was significantly lower in CWD patients than in control subjects ($P < 0.0001$ for all stimuli) (Fig. 1C). In contrast to whole-blood stimulation, we observed a modest CD4$^+$ T cell reactivity to EC-Hsp70 in thawed PBMC of control subjects, which was higher ($P = 0.0010$) than that in PBMC from CWD patients but lower ($P = 0.030$) than that of CD4$^+$ T cell reactivity toward TW-Hsp70 in control PBMC. The reactivity of CD4$^+$ T cells in thawed PBMC from both CWD patients and control subjects to the control protein EC-GrpE or MT-GrpE was similar and very low. In CWD patients, the reactivity to EC-GrpE was reduced compared to the reactivity to TW-GrpE ($P = 0.021$), and healthy individuals revealed a reduced reactivity to GrpE of *M. tuberculosis* and *E. coli* compared to TW-GrpE ($P = 0.006$ and $P = 0.045$, respectively).

As Hsp70 and GrpE of *T. whipplei* were able to induce recall memory responses in CD4$^+$ T cells of healthy subjects *in vitro*, these proteins represent CD4$^+$ T-cell antigens. Corresponding to the previously described reduced reactivity of peripheral and mucosal CD4$^+$ T cells of CWD patients to *T. whipplei* lysate (16), these antigenic proteins did not elicit sufficient CD4$^+$ T-cell responses in CWD patients.

Patients with active CWD reveal an overall enhanced activation of blood CD8$^+$ T cells. To determine the cytolytic potential of T cells, the expression of granzyme B and perforin, as surrogate markers for the cytotoxic capacity, was directly analyzed in CD8$^+$ T cells of fresh whole blood (Fig. 2). The percentage of granzyme B$^+$ cells within the CD8$^+$ CD3$^+$ T-cell population of peripheral blood was enhanced in untreated and treated CWD patients compared to that of healthy controls ($P < 0.001$ for both)

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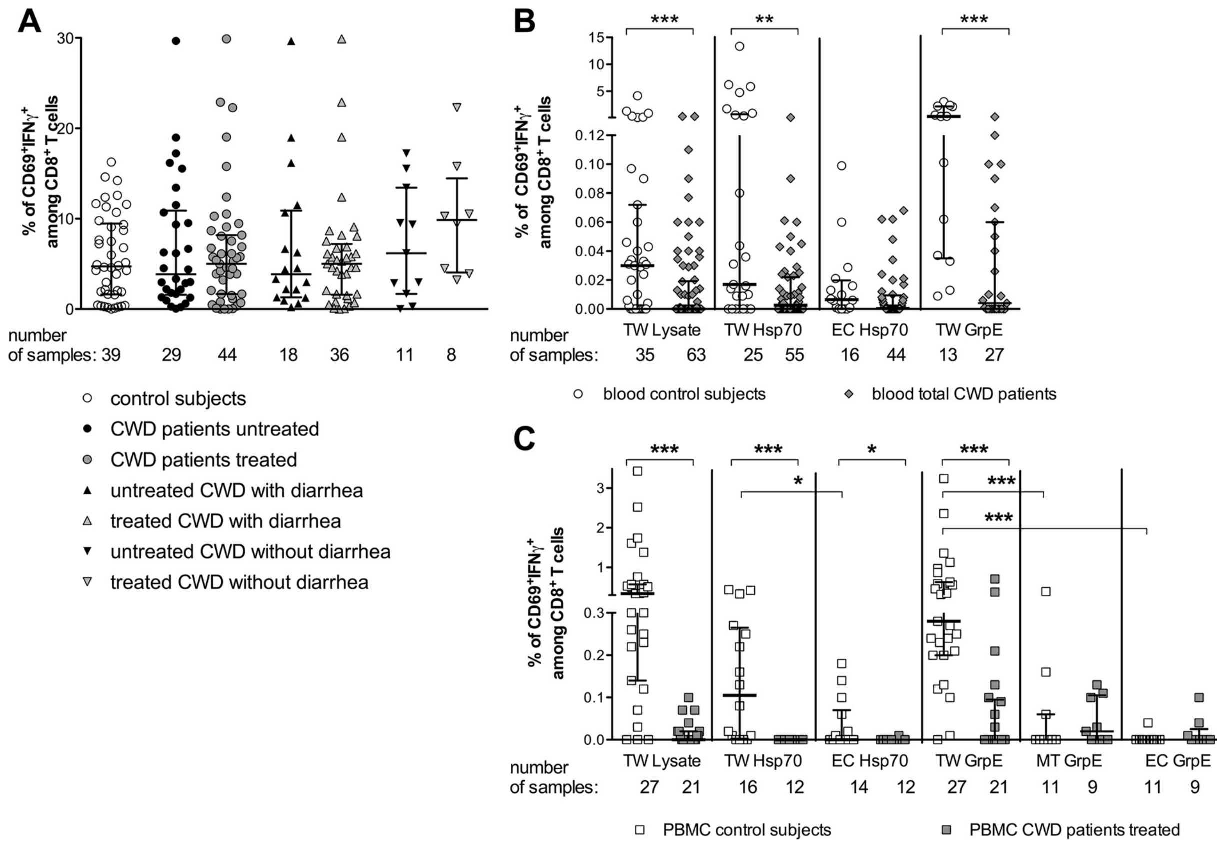

FIG 3 $CD8^+$ T-cell reactivity from fresh whole blood cells and cryopreserved PBMC. Whole blood (A and B) and PBMC (C) of CWD patients and healthy controls were stimulated as described for Fig. 1. Individual percentages and median values of $CD69^+$ IFN-γ^+ cells among $CD8^+$ T cells upon stimulation of whole blood with SEB (A) or upon stimulation of whole blood (B) and thawed PBMC (C) of both CWD patients and control subjects with *T. whipplei* antigens as analyzed by flow cytometry. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ by Mann-Whitney *U* test.

(Fig. 2A). The portion of perforin$^+$ granzyme B$^+$ cells among $CD8^+$ $CD3^+$ T cells was enhanced in untreated CWD patients compared to that of healthy subjects ($P < 0.001$) (Fig. 2B). Corresponding to the fact that the unspecific $CD4^+$ T-cell activation by SEB normalized during the course of treatment for CWD, the percentages of granzyme B$^+$ cells among $CD8^+$ T cells ($P = 0.008$) (Fig. 2A) and perforin$^+$ granzyme B$^+$ cells among $CD8^+$ T cells ($P = 0.008$) (Fig. 2B) decreased significantly.

As a more functional marker for cytotoxic activity of $CD8^+$ T cells, the expression of CD107a (LAMP-1), essential for degranulation (32), was determined after stimulation with SEB and *T. whipplei* lysate. While upon stimulation with SEB the upregulation of CD107a was similar in healthy subjects and treated and untreated CWD patients (Fig. 2C), stimulation with *T. whipplei* lysate induced an elevated percentage of CD107a$^+$ $CD8^+$ cells in untreated CWD patients ($P = 0.040$) (Fig. 2D).

Thus, contact with *T. whipplei* stimulates cytotoxic activity, and the enhanced proportion of potentially cytotoxic $CD8^+$ T cells before the onset of treatment was opposed to generally insufficient $CD4^+$ T-cell reactivity in CWD patients.

$CD8^+$ T-cell reactivity to recombinant *T. whipplei* proteins is reduced in CWD patients. In order to explore if the enhanced cytotoxic potential of $CD8^+$ T cells reflected *T. whipplei*-specific expression of IFN-γ, $CD8^+$ T cells in whole blood of patients with CWD were analyzed for their specific reactivity. In contrast to the diminished $CD4^+$ T-cell reactivity to SEB in active disease (Fig. 1A), the reactivity of $CD8^+$ T cells was affected by neither the disease itself nor the treatment status or the disease manifestation of CWD patients (Fig. 3A). Correspondingly, the $CD8^+$ T-cell reactivity to *T. whipplei* lysate and recombinant Hsp70 or GrpE of *T. whipplei* proteins did not change following treatment of CWD ($P = 0.17$ according to Kruskal-Wallis test)

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(Fig. S3A), and the activation following incubation with *T. whipplei* lysate was not significantly influenced by the clinical manifestation (patients with diarrhea versus patients without diarrhea) (Fig. S3A). Also, neither CNS infection nor the presence or absence of HLA A2 significantly influenced $CD8^+$ reactivity.

As for $CD4^+$ T cells, the reactivity of $CD8^+$ T cells of CWD patients matched before and after treatment did not reveal significant differences ($n = 11$ for *T. whipplei* lysate; $n = 5$ for TW-Hsp70; $n = 4$ for EC-Hsp70) (Fig. S3B). Therefore, we again merged the data sets from untreated and treated CWD patients in the following analyses. As earlier shown for $CD4^+$ T cells, percentages of antigen-specific IFN-γ-producing activated $CD8^+$ T cells upon stimulation with *T. whipplei* lysate or with Hsp70 or GrpE of *T. whipplei* were significantly reduced in CWD patients compared to those of healthy control subjects (Fig. 3B). Also similar to $CD4^+$ T cells (Fig. 1B), the proportion of $CD8^+$ T cells reacting to EC-Hsp70 was low in all groups (Fig. 3B). $CD8^+$ T cells of control subjects showed a prominent reactivity toward Hsp70 or GrpE of *T. whipplei* that was significantly higher than that of the $CD8^+$ T cells from CWD patients (Fig. 3B).

The reactivity of $CD8^+$ T cells from fresh whole blood from CWD patients and healthy subjects to GrpE of *T. whipplei* was significantly higher than that of $CD4^+$ T cells ($P = 0.021$ and $P = 0.024$, respectively, by Wilcoxon matched-pairs signed-rank test) (Fig. 1B). In addition, CWD patients revealed an enhanced reactivity of $CD8^+$ T cells compared to $CD4^+$ T cells to TW-Hsp70 ($P = 0.026$ by Wilcoxon matched-pairs signed-rank test) (Fig. 1B).

As for the $CD4^+$ T cells, stimulation of fresh whole blood and thawed PBMC of CWD patients and control subjects similarly activated $CD8^+$ T cells. As an example, the reactivity to TW-Hsp70 and SEB is presented in Fig. S4. Upon stimulation of freshly thawed cryopreserved PBMC with *T. whipplei* lysate or Hsp70 or GrpE of *T. whipplei*, the percentage of *T. whipplei*-specific $CD69^+$ IFN-γ^+ cells among $CD8^+$ T cells was significantly lower in CWD patients than in healthy individuals (Fig. 3C) according to the results from $CD4^+$ T cells (Fig. 1). Again corresponding to data of $CD4^+$ T cells, thawed PBMC but not fresh whole blood of control subjects showed a low reactivity of $CD8^+$ T cells to EC-Hsp70 that was significantly higher than the specific reactivity to EC-Hsp70 in CWD patients ($P = 0.041$) but significantly reduced compared to the $CD8^+$ reactivity to TW-Hsp70 in controls ($P = 0.032$). $CD8^+$ T cells in all samples of thawed PBMC almost did not respond to the control GrpE of *M. tuberculosis* or *E. coli*. In healthy control subjects, the reactivity to GrpE of *M. tuberculosis* and *E. coli* was reduced compared to that to TW-GrpE ($P < 0.001$ for both).

Recombinant Hsp70 and GrpE of *T. whipplei* stimulated peripheral $CD8^+$ T cells of control subjects. Although the elevated overall peripheral $CD8^+$ T-cell activation and *T. whipplei*-specific degranulation, indicated by CD107a expression in untreated CWD, did not translate into *T. whipplei*-specific $CD8^+$ T-cell responses, the reactivity to TW-Hsp70 and TW-GrpE was more pronounced in $CD8^+$ than in $CD4^+$ T cells.

DISCUSSION

Accumulating evidence suggests that *T. whipplei* causes acute infections, including bacteremia and gastroenteritis, and local chronic infections without gastrointestinal involvement, such as endocarditis, uveitis, or pneumonia, that do not necessarily lead to full-blown CWD (33, 34). Only a small number of predisposed individuals develop CWD upon infection with *T. whipplei*, when the bacterium is not efficiently eliminated due to immunological host factors related to antigen processing or presentation as, for example, a CWD-associated HLA haplotype and alternative macrophage activation (11, 35). A reduced specific reactivity of CWD patients compared to control subjects to lysate of *T. whipplei* Twist-Marseille has previously been demonstrated for peripheral as well as mucosal $CD4^+$ T cells (16), and *T. whipplei* Twist strains Neuro1, Endo5, and Slow2 were not able to stimulate T cells of CWD patients (unpublished data). A proteomic approach identified *T. whipplei* antigens for the development of a serological test for CWD; among these, the Hsp70 cofactor GrpE appeared to be one of the most suitable candidate proteins (22).

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The direct role of Hsp70 as an antigen is well explained by the fact that heat shock proteins are abundant in the pathogen, especially under stress conditions, and, due to their high conservation among various pathogenic and nonpathogenic bacteria, immunologic memory for conserved heat shock proteins is generated during life by subsequent encounter with microbes (24). Hsp70 was established as a common antigen of *Leishmania infantum* (25) and *Helicobacter pylori* (26), and the Th2 response to Hsp70 of *Salmonella typhi* protects mice from lethal *Salmonella* infection (30). Tumor vaccination strategies for human papillomavirus 16 benefit from the adjuvant-type capacity of Hsp70 by fusing it to mesothelin or the oncoprotein E7 (27, 28), and the complexing of potential antigens with Hsp70 or GroEL provides immunogens for CD8$^+$ T-cell activation (29).

Utilizing recombinant purified proteins, we tested the antigenic capacity of Hsp70 and GrpE of *T. whipplei* to activate peripheral CD4$^+$ and CD8$^+$ T cells of CWD patients and healthy individuals. This approach demonstrated that in immunocompetent persons, Hsp70 itself and its cofactor GrpE of *T. whipplei* can induce strong specific immune responses that are absent from patients suffering from CWD. In contrast to Hsp70 of *T. whipplei*, Hsp70 of *E. coli* and GrpE of *M. tuberculosis* and *E. coli* had only a low antigenic potential. This demonstrated that humans are tolerized to *E. coli* proteins due to frequent contact with the commensal intestinal bacterium (36) and that subjects recruited for this study were not infected by *M. tuberculosis*. In terms of their potential to stimulate specific CD4$^+$ and CD8$^+$ T-cell reactions, only recombinant Hsp70 and GrpE from *T. whipplei* mimicked the effect of the complete *T. whipplei* lysate. The noticeable conservation of heat shock proteins of *T. whipplei* guarantees stable immunity even if different strains of *T. whipplei* elicited the initial immune response. While Hsp70 of *T. whipplei* Twist is 100% identical in 18 of 19 published strains, GrpE of *T. whipplei* Twist is identical in four additional strains and shows 97 to 99% similarity to GrpE of 14 other strains from the available sequence data. In contrast, the similarity to Hsp70 and GrpE of *E. coli* and GrpE of *M. tuberculosis* is only low, and the majority of putative T cell epitopes is unique for the *T. whipplei* proteins. Thus, interspecies cross-reactivity is improbable.

Illustrating a specific aspect of CWD development, CWD patients were not able to induce proinflammatory CD4$^+$ T-cell responses to recombinant GrpE and Hsp70 of *T. whipplei*. This lack of a proper response seems independent of clinical manifestation of CWD and was not reverted by successful treatment. The frequencies of CD4$^+$ and CD8$^+$ T cells expressing the general activation marker CD69 or the frequency of CD4$^+$ T cells expressing CD40L, a marker of CD4$^+$ T cells specifically activated by an antigen, remained low in CWD patients compared to that of healthy controls. Thus, reduced Th1 reactivity is explained not only by specifically repressed IFN-γ expression but also by an overall insufficient T-cell activation. Since the infecting *T. whipplei* strain does not influence the manifestation of the disease (9), the immunological dysfunctions of antigen-presenting cells of CWD patients and the modulation of regulatory T cells imply that the immunologic reaction of the host is responsible for the tolerance of CD4$^+$ T cells (16, 37–39). However, while CWD treatment status does not influence the reactivity to *T. whipplei* antigens, the T cell reaction to SEB is influenced by the clinical manifestation and the treatment status. In CWD patients with diarrhea, continuous microbial translocation across a leaky gut (40) probably exhausts T cells in addition to the chronic infection, resulting in a lower reactivity to SEB.

However, peripheral CD8$^+$ T cells seemed at least in part to balance the inability of CD4$^+$ T cells to combat *T. whipplei*. In untreated CWD patients, CD8$^+$ T cells reveal an enhanced cytolytic potential and *T. whipplei*-specific degranulation. CD8$^+$ T cells of all CWD patients showed an improved reactivity to recombinant Hsp70 and GrpE of *T. whipplei* compared to CD4$^+$ T cells. Thus, CD8$^+$ T cells might, at least in part, assume the defense against *T. whipplei*. However, since neither the CD8$^+$ intraepithelial T cell level nor perforin expression in the duodenal mucosa is enhanced (18, 40), CD8$^+$ T-cell activation seems to be restricted to the peripheral blood and thus might not effectively assist mucosal defense against *T. whipplei*. Pointing to potential differential mecha-

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nisms to activate human peripheral memory T cells, soluble heat shock proteins from *Burkholderia pseudomallei* directly induce granzyme B production in CD8$^+$ T cells *in vitro*, while sufficient antigen-specific CD4$^+$ T-cell activation depends on direct contact between monocyte-derived dendritic cells and memory T cells (41). In combination with the impaired monocyte function and reduced interleukin-12 (IL-12) production in CWD patients (18, 21, 42), we assumed that Hsp70 and GrpE, showing comparable activation patterns in T cells, were potent immunogens of *T. whipplei* directly acting as antigenic structures. The protective cellular immunity directed against *T. whipplei* and, more specifically, against Hsp70 and GrpE in subjects not affected by CWD probably evolves during the first contact with the agent that is supposed to occur in early childhood and may result in self-limiting gastroenteritis (10).

It was previously shown that the T-cell hyporesponsiveness of peripheral and mucosal CD4$^+$ T cells of CWD patients seems to be restricted to selected stimuli and to *T. whipplei* itself (16). A lack of inflammatory activation of antigen-presenting cells, proven previously by the presence of alternatively activated macrophages in CWD patients, contributes to deficient T-cell reactions in CWD patients. Deficient antigen presentation, lack of costimulation, and the absence of an inflammatory response against *T. whipplei* might further impair T-cell activation and allow the establishment of a chronic infection in predisposed individuals (11, 16, 18, 21).

In summary, we have detected a reduced specific reactivity against two defined proteins of *T. whipplei* in CWD patients, while peripheral T cells of healthy individuals reveal a strong specific response that might be important for the normal lifelong protection from CWD. Since these proteins mimic the effects of whole *T. whipplei* lysate and our T-cell stimulation system using recombinant Hsp70 and GrpE from *T. whipplei* worked with fresh and cryopreserved peripheral blood samples, we conclude that Hsp70 and its cofactor, GrpE, can be considered immunogenic proteins. In the future, the analysis of T-cell reactivity to Hsp70 and GrpE of *T. whipplei* might be of diagnostic value, since it enables the discrimination of healthy subjects from CWD patients.

MATERIALS AND METHODS

Patients and control subjects. We studied specimens of 71 patients with different stages of CWD and of 94 healthy volunteers (Table 1). CWD patients were diagnosed from 2002, and specimens were collected consecutively from the end of 2008 until 2014. Where appropriate, we included the tests available at the time of sampling. Recombinant proteins were expressed successively, and lower numbers of specimens were stimulated with the proteins produced last. All patients were affected by CWD, as confirmed by the presence of a typical histology showing periodic acid-Schiff$^+$ macrophages infiltrating the duodenal mucosa in combination with positive *T. whipplei*-specific PCR and/or immunohistochemistry (2). However, concerning the clinical manifestations, the 55 patients included in the whole-blood stimulation assay differed: diarrhea was observed in 41 patients, while 14 did not suffer from gastrointestinal symptoms; 18 patients with infection of the CNS, 17 patients without CNS infection, and 20 with unknown CNS status were included. Of 22 patients tested for the presence of HLA-A2, 13 were positive and nine negative.

This study was approved by the local ethics committee of the Charité, and all subjects gave their written consent to participate (approval number EA4/122/10).

Cloning of *hsp70* and *grpE* from *T. whipplei* for recombinant expression. *T. whipplei* strain Twist-Marseille was cultured in a cell-free system as previously described (43). Genomic DNA of *T whipplei* Twist and *Escherichia coli* BL21(DE3)pLysS (Life Technologies, Regensburg, Germany) was prepared from bacteria (10^9/ml) according to the manufacturer's instructions (Zymo Research, Freiburg, Germany). Specific DNA for *dnaK-hsp70* and *grpE* of *T. whipplei* or of *E. coli* was retrieved by standard PCR (Life Technologies) with primer pairs designed from the *T. whipplei* Twist sequence (NCBI accession number NC_004572) and from the *E. coli* K-12 MG1655 sequence (NCBI accession number NZ_CP014225.1). The internal EcoRI site in *E. coli dnaK* was inactivated by *in vitro* mutagenesis not affecting the amino acid sequence. All primers were purchased from TIB Molbiol (Berlin, Germany) (Table 2). For the expression of GrpE, the sequence from *Mycobacterium tuberculosis* Erdman (NCBI accession number AP012340.1, nucleotides 422.077 to 422.784) was transformed in a codon-adapted nucleotide sequence for expression in *E. coli* and synthesized, including the terminal restriction sites for EcoRI and SmaI (GeneCust, Dudelange, Luxemburg). Verified sequences (Seqlab, Göttingen, Germany) for all proteins were cloned into the expression vector pGEX-2T (GE Healthcare, Freiburg, Germany) in frame to glutathione *S*-transferase (GST).

Production of Hsp70 and GrpE as GST fusion proteins. If not otherwise noted, all reagents were purchased at the highest purity available from Merck (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany), and all steps were performed at 4°C. *E. coli* BL21(DE3)pLysS was transformed with pGEX-2T coding for GST-Hsp70 or GST-GrpE fusion proteins, as well as for free GST as a control.

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TABLE 1 Characteristics of CWD patients and healthy controls

Subject type	No. of specimens or subjects, with gender and age[a]				
	Total	Heparinized blood for antigen-specific stimulation	Heparinized blood for CD107 analysis	Heparinized blood for phenotyping	Cryopreserved PBMC
CWD	102 samples from 71 patients (19 F, 52 M; mean age, 57 yr; range, 31–73 yr); 31 patients before and after treatment; 6 patients untreated; 34 patients treated	67 samples from 55 patients (19 F, 36 M; mean age, 57 yr; range, 31–73 yr); 12 patients before and after treatment; 15 patients untreated; 28 patients treated	26 samples from 25 patients (4 F, 21 M; mean age, 57 yr; range, 41–80); 1 patient before and after treatment; 4 patients untreated; 20 patients treated	50 samples from 38 patients (7 F, 31 M; mean age, 55 yr; range, 41–80 yr); 12 patients before and after treatment; 4 patients untreated; 22 patients treated	21 samples from 21 patients (7 F, 14 M; mean age, 59 yr; range, 45–73 yr); 21 patients treated
Control	94 subjects (44 F, 50 M; mean age, 49 yr; range, 22–88 yr)	40 subjects (18 F, 22 M; mean age, 51 yr; range, 22–88 yr)	22 subjects (13 F, 9 M; mean age, 51 yr; range, 24–81 yr)	54 subjects (27 F, 27 M; mean age, 49 yr; range, 24–84 yr)	27 subjects (12 F, 15 M; mean age, 43 yr; range, 22–88 yr)

[a]F, female; M, male.

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TABLE 2 Primers for cloning of *T. whipplei* and *E. coli* genes in pGEX-2T

Primer	Sequence	Restriction site
TWT750/*dnak*	5′-GCAAGGGGATCCATGTCAAGAGCTGTTGG-3′	BamHI
	5′-CCTGTTTAAGGAATTCACTATTTAG-3′	EcoRI
TWT749/*grpE*	5′-GGATCCACTAAGTCAAACGAGACTG-3′	BamHI
	5′-GAATTCACCATTCGGAACCTGTAC-3′	EcoRI
EC/*dnak*	5′-GTGGAGACGTTGGATCCGGTAAAATAATTGG-3′	BamHI
	5′-CCGTTTATAGGGGAATTCTTTTTTGTC-3′	EcoRI
EC/*grpE*	5′-CCCGGGGAGTAGTAAAGAACAG-3′	SmaI
	5′-GAATTCAGCTTTTGCTTTCG-3′	EcoRI

Recombinant protein production was induced by isopropyl-β-D-1-thiogalactopyranoside (200 μg/ml) for 4 h. Bacteria were lysed by sequential freeze-thawing cycles and intermittent mild ultrasound (30%; Bandelin, Berlin, Germany) in the presence of lysozyme, DNase I, and a proteinase inhibitor cocktail containing aprotinin, leupeptin, Pefabloc, and EDTA. Clear bacterial lysates were subjected to glutathione affinity chromatography (GST Trap; GE Healthcare), bound proteins were eluted by reduced glutathione (500 mmol/liter), and the buffer was exchanged for phosphate-buffered saline (PBS) by gel filtration (Sephadex G-25; GE Healthcare). The purity of the protein preparations was assessed by denaturing polyacrylamide gel electrophoresis and Coomassie brilliant blue R-250 staining; protein concentrations were determined by the bicinchoninic acid method (Thermo Pierce, Rockford, IL). Due to native stable complexes, preparations of recombinant *E. coli* GrpE contained natural Hsp70 of the expression host (estimated ratio, 100:1). The level of bacterial endotoxin was lower than 0.1 EU/ml as determined by the LAL chromogenic endpoint assay (HyCult, Uden, The Netherlands). Aliquots were shock-frozen over liquid nitrogen and stored at −80°C.

Isolation of peripheral cell populations. Fresh blood samples were collected in sodium-heparinized tubes (Vacutainer; BD Biosciences, Heidelberg, Germany) and processed within 24 h. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Paque (GE Healthcare) in Leucosep tubes (Greiner Bio-One, Kremsmünster, Austria) and stored in freezing medium containing fetal calf serum (FCS) and dimethyl sulfoxide (10%) over liquid nitrogen until assay. Before stimulation, PBMC were rapidly thawed at 37°C, washed two times in culture medium [RPMI 1640 with 10% FCS, 25 mmol/liter 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 4 mmol/liter L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin] and resuspended at 2×10^7 cells/ml.

Stimulation of PBMC and whole blood. General Th1 reactivity in fresh heparinized blood and PBMC was determined after short-term culture at 37°C in a humidified 5% CO_2 air atmosphere as previously described (44). PBMC (2×10^6 cells/ml) were stimulated in culture medium with reduced FCS (5%) in the presence of anti-CD28 (1 μg/ml; CD28.2; BD Biosciences), and whole blood was stimulated in the presence of anti-CD28 (CD28.2) and anti-CD49d (9F10; each at 1 μg/ml; BD Biosciences). The following substances for stimulation were used: lysate of heat-inactivated *T. whipplei* Twist-Marseille from culture in axenic medium (10^7 bacteria/ml) as previously described (3, 43), recombinant GST fusion proteins of GrpE from *T. whipplei* (TW-GrpE), *E. coli* (EC-GrpE) and *M. tuberculosis* (MT-GrpE; all at 50 μg/ml), and GST fusion proteins of Hsp70 from *T. whipplei* (TW-Hsp70; 80 μg/ml) and *E. coli* (EC-Hsp70; 33 μg/ml). *Staphylococcus* enterotoxin B (SEB; 2 μg/ml, Sigma-Aldrich, Taufkirchen, Germany) served as a positive control. Negative controls contained no supplement or recombinant protein-free GST (22 μg/ml). Brefeldin A was added for the last 3 h of stimulation to detect intracellular IFN-γ. After incubation, cells were fixed with paraformaldehyde (4%) in PBS, washed, and resuspended in PBS containing bovine serum albumin (5 mg/ml) and NaN_3 (200 μg/ml). Cell suspensions were stored at 4°C before intracellular staining and flow-cytometric analysis.

Analysis of CD107a expression. For the analysis of CD107a expression, as a functional marker of cytotoxic activity (32), fresh whole blood was mixed with anti-CD107a (H4A3; BD Biosciences) in the presence of anti-CD28 and anti-CD49d with either lysate of heat-inactivated *T. whipplei* Twist-Marseille or SEB as a positive control as described above. The negative control contained no supplement. After 1 h at 37°C in a humidified 5% CO_2 air atmosphere, brefeldin A (10 μg/ml) and monensin (0.67 μl/ml; both from Sigma-Aldrich) were added. Subsequently, samples were incubated for a further 4 h (37°C, 5% CO_2), followed by surface staining of CD3 and CD8, lysis of erythrocytes, and fixation as described below.

Flow-cytometric analysis. Antigen-specific $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry as previously described (16, 38, 44) with antibodies against CD3 (SK7), CD4 (RPA-T4), CD8 (SK1), and IFN-γ (B27) from BD Biosciences, as well as against CD69 (TP1.55.3; Beckman Coulter, Krefeld, Germany) and CD40L (24-31; e-Bioscience, Frankfurt, Germany). The phenotype of $CD8^+$ T cells was determined by staining of fresh whole blood with anti-CD3 and anti-CD8, subsequent lysis of erythrocytes with fluorescence-activated cell sorting (FACS) lysing buffer (BD Biosciences), and fixation with 4% paraformaldehyde (Sigma-Aldrich). For detection of intracellular surrogate markers of cytotoxicity, cells were permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with anti-perforin (δG9) and anti-granzyme B (GB11) antibodies (both BD Biosciences). Data were acquired on a FACSCalibur or FACSCanto II (both BD Biosciences) and analyzed with FlowJo software (FlowJo, Ashland, OR) (for the gating strategy, see Fig. S1 and S2 in the supplemental material). Background levels, as determined from parallel cultures that

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did not receive any of the stimuli, as outlined above, or free GST for the GST fusion proteins, were subtracted from the values obtained after stimulation with *T. whipplei* lysate, recombinant proteins, or SEB. Values below the background level were defined as 0.

Statistical analysis. Data were analyzed using GraphPad Prism 5 software (GraphPad, La Jolla, CA). Quantitative measurements are presented as individual data points with medians. Groups were compared by Kruskal-Wallis test followed by Mann-Whitney *U* test or Wilcoxon matched-pair signed-rank test for paired observations. Two-tailed *P* values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00363-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 2, PDF file, 1.7 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 4, PDF file, 3.5 MB.

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