**Brucella melitensis** T Cell Epitope Recognition in Humans with Brucellosis in Peru

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**Brucella melitensis**, one of the causative agents of human brucellosis, causes acute, chronic, and relapsing infection. While T cell immunity in brucellosis has been extensively studied in mice, no recognized human T cell epitopes that might provide new approaches to classifying and prognosticating *B. melitensis* infection have ever been delineated. Twenty-seven pools of 500 major histocompatibility complex class II (MHC-II) restricted peptides were created by computational prediction of promiscuous MHC-II CD4+ T cell derived from the top 50 proteins recognized by IgG in human sera on a genome level. *B. melitensis* protein microarray. Gamma interferon (IFN-γ) and interleukin-5 (IL-5) enzyme-linked immunospot (ELISPOT) analyses were used to quantify and compare Th1 and Th2 responses of leukapheresis-obtained peripheral blood mononuclear cells from Peruvian subjects cured after acute infection (n = 9) and from patients who relapsed (n = 5). Four peptide epitopes derived from 3 *B. melitensis* proteins (BMEI 1330, a DegP/HtrA protease; BMEII 0029, type IV secretion system component VirB5; and BMEII 0691, a predicted periplasmic binding protein of a peptide transport system) were found repeatedly to produce significant IFN-γ ELISPOT responses in both acute-infection and relapsing patients; none of the peptides distinguished the patient groups. IL-5 responses against the panel of peptides were insignificant. These experiments are the first to systematically identify *B. melitensis* MHC-II-restricted CD4+ T cell epitopes recognized by the human immune response, with the potential for new approaches to brucellosis diagnostics and understanding the immunopathogenesis related to this intracellular pathogen.

**Brucella melitensis** is a Gram-negative alphaproteobacterium that is one of the causative agents of brucellosis, a common bacterial zoonosis worldwide and a potential biological warfare agent. Humans may become infected by *B. melitensis* by ingesting unpasteurized dairy products of goats, sheep, and camels, via occupational contact with these animals (abattoir workers, dairy farmers, veterinarians), or by inhalation in the microbiology laboratory (1–3). Among the *Brucella* species affecting humans, *B. melitensis* is the most virulent and is associated with the relapsing form of the disease despite adequate antibiotic therapy (4). In areas of brucellosis endemicity, diagnosis is usually established serologically based on agglutination of fixed *Brucella abortus* as antigen (2). Relapsing brucellosis may be difficult to distinguish using serological tests; culture isolation is the gold standard. The clinical manifestations of brucellosis such as undulant fever, focal pyogenic infection, and even the chronic fatigue syndrome-like illness of chronic brucellosis seem to be immune mediated (5), related to cytokine production, and an ineffective cell-mediated immune response.

Several animal models (naturally infected hosts [cattle, goats], mouse models, and human infection) have been used to investigate acquired immune responses to brucellosis (6–8) and have further provided elucidation of mechanisms that allow *Brucella* spp. to evade cell-mediated immune responses (9, 10). The clinical manifestations of brucellosis after exposure are usually initiated by cytotoxic immune responses, as surges of cytokines are associated with the destruction of *Brucella* within infected macrophages. Currently, the mechanisms by which cell-mediated immune responses confer protection and those leading to disease manifestations are unexplained; especially unknown are the reasons why some individuals do not have complications or continuation of acute brucellosis and some develop relapse (11, 12).

After first encountering *Brucella* antigens, antigen-presenting cells (APCs) produce interleukin-1 (IL-1), IL-6, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ), initiating innate immune responses (including natural killer cells) that may limit the initial spread of the organism. Infected APCs in which *Brucella* organisms are residing within unactivated phagosomes are likely to present some subset of (unknown) pepticid *Brucella* antigens to CD4+ and CD8+ cells, inducing a Th1 response associated with IFN-γ release. Furthermore, it has been demonstrated that human dendritic cell maturation can be down-regulated by *Brucella suis*, by limiting TNF-α secretion (13). It appears that *Brucella* spp. are able to regulate cytokine production, initially demonstrating a Th1 response and then gradually shifting toward a Th2 response over time and during intracellular parasitism, thereby depressing the human cellular immune response (11, 13).
The functional consequences of antigen-specific IFN-γ release are unclear but do not lead to elimination of organisms during active, symptomatic infection, and it likely results in clinical symptomatology (i.e., fever, sweating, and weight loss). Clonal T-cell expansion is initiated with production of IL-2 and IL-12, which initiates a CD8⁺ cytotoxic response on Brucella-infected cells. Infected macrophages produce IL-12 and IFN-γ, which regulate antigen presentation and may contribute to the limitation of intracellular bacterial replication through unknown mechanisms (15). Data also suggest that Brucella spp. also modify the initial immune response once phagocytosed into APCs, mainly because of the inability of macrophages and dendritic cells to display Brucella antigens via major histocompatibility complex class I (MHC-I) molecules, and therefore decreasing the CD8⁺ T cell response (16). Alteration of T-cell function may be the key to explaining the clinicopathological manifestations of relapsing brucellosis. Based on these considerations, the unusual and diverse manifestations of relapsing brucellosis could be related to several potential immunopathogenic mechanisms: an ineffective CD4⁺ effector response, a downregulated CD8⁺ T cell response, or a continued, established Th2 response, each of which could result in an incomplete resolution of the infection.

A comprehensive systems biology analysis of human antibody responses in acute B. melitensis brucellosis in Peru was recently reported (17, 18). A collection of sera isolated from individuals from one of the following groups was used to probe large-scale B. melitensis protein microarrays, including an ∼1,400-protein array and a 3,300-protein array representing nearly the entire encoded proteome: Brucella blood culture positive, blood culture negative with positive Rose Bengal (RB), blood culture negative with negative Rose Bengal, and two naive groups (from both U.S. and Peruvian individuals) (18). Sets of proteins that differentiated acutely infected from uninfected patient groups that were recognized by patient IgG responses were identified. This broad spectrum of antibody responses demonstrated the differences between the manners in which these groups respond to Brucella protein epitopes. IgG responses reflect antigen-specific, cognate, CD4⁺ T cell-regulated antibody class switching and affinity maturation. Hence, we sought to identify potential peptide MHC-II-restricted CD4⁺ T cell epitopes that might correlate with brucellosis relapse. While there have been analyses of specific Brucella proteins producing an IFN-γ response (19) and an experimental IFN-γ release assay has even been developed for ovine brucellosis (20), there has not, however, been one developed for human infection to differentiate immune responses associated with different forms of brucellosis, to definitely diagnose previous exposure or identify targets of protective immunity. T cell epitopes of Brucella spp. recognized by human immune responses have not been described to date (21).

In this study, we sought to test the hypothesis that recovered acute-brucellosis patients and relapsing patients responded differentially to predicted promiscuous MHC-II B. melitensis epitopes. We tested this hypothesis by in silico prediction of CD4⁺ T cell epitopes from the top 50 proteins recognized by human IgG responses as determined by previous genome level protein microarray analyses (17, 18). These pepitide epitopes were used to characterize the cellular immune responses of brucellosis patients and to compare these cytokine responses (Th1/Th2) (15) of both recovered acute-disease and chronic relapsing patients.

### MATERIALS AND METHODS

**Human subjects.** This study was approved by the Humans Research Protections Program of the University of California, San Diego (UCSD), and the Comités de Ética of the Universidad Peruana Cayetano Heredia (UPCH) and Hospital Nacional Cayetano Heredia, Lima, Peru. This study was also reviewed and determined to be acceptable by the Peruvian Ministry of Health, National Institute of Health, Lima, Peru.

Patients were enrolled primarily from referrals of potential brucellosis patients from the Hospital Nacional Cayetano Heredia, Lima, Peru. These referred patients were part of a prospective study of brucellosis from 2005 to 2010 that involved screening of more than 1,000 referred patients for brucellosis. Patients referred for suspicion of brucellosis underwent serological screening using the qualitative Rose Bengal agglutination test; RB-positive patients underwent further diagnostic testing. Thirteen subjects confirmed to have acute B. melitensis infection based on positive B. melitensis blood culture or a tube agglutination titer of ≥1/160 were enrolled. One additional subject, positive by blood culture and who had a tube agglutination titer of 1:320, was recruited at UCSD. Overall, 14 subjects had brucellosis, 9 of whom had a single episode of acute, blood culture-positive infection, and 5 of whom had symptomatic or serologically or culture-confirmed relapse. Relapse was defined serologically as a 4-fold rise in titer compared to previous recovery from acute illness or as

**TABLE 1 Brucella melitensis-infected patient demographics**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Infection type</th>
<th>Age at diagnosis (yr)</th>
<th>Diagnosis method</th>
<th>Diagnosis date(s)</th>
<th>Leukapheresis date</th>
<th>PBMC viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acute</td>
<td>F</td>
<td>30</td>
<td>Culture</td>
<td>11/19/2007</td>
<td>10/23/2010</td>
</tr>
<tr>
<td>2</td>
<td>Acute</td>
<td>F</td>
<td>42</td>
<td>Culture</td>
<td>12/01/2007</td>
<td>04/06/2011</td>
</tr>
<tr>
<td>3</td>
<td>Acute</td>
<td>F</td>
<td>51</td>
<td>Rose Bengal</td>
<td>06/01/2006</td>
<td>10/30/2010</td>
</tr>
<tr>
<td>4</td>
<td>Acute</td>
<td>F</td>
<td>27</td>
<td>Culture</td>
<td>08/29/2008</td>
<td>02/18/2011</td>
</tr>
<tr>
<td>5</td>
<td>Acute</td>
<td>F</td>
<td>61</td>
<td>Culture</td>
<td>09/02/2008</td>
<td>11/20/2010</td>
</tr>
<tr>
<td>6</td>
<td>Relapse</td>
<td>F</td>
<td>57</td>
<td>Culture</td>
<td>A, 10/02/2007</td>
<td>R, 01/12/2008</td>
</tr>
<tr>
<td>7</td>
<td>Relapse</td>
<td>M</td>
<td>37</td>
<td>Culture</td>
<td>A, 09/17/2009</td>
<td>R, 01/05/2010</td>
</tr>
<tr>
<td>8</td>
<td>Relapse</td>
<td>M</td>
<td>42</td>
<td>Culture</td>
<td>A, 01/02/2010</td>
<td>R, 06/10/2010</td>
</tr>
<tr>
<td>9</td>
<td>Relapse</td>
<td>M</td>
<td>64</td>
<td>Culture</td>
<td>A, 07/02/2011</td>
<td>R, 03/18/2012</td>
</tr>
</tbody>
</table>

⁷Patient was phlebotomized, not leukapheresed.

\(14\). The functional consequences of antigen-specific IFN-γ release are unclear but do not lead to elimination of organisms during active, symptomatic infection, and it likely results in clinical symptomatology (i.e., fever, sweating, and weight loss). Clonal T-cell expansion is initiated with production of IL-2 and IL-12, which initiates a CD8⁺ cytotoxic response on Brucella-infected cells. Infected macrophages produce IL-12 and IFN-γ, which regulate antigen presentation and may contribute to the limitation of intracellular bacterial replication through unknown mechanisms (15). Data also suggest that Brucella spp. also modify the initial immune response once phagocytosed into APCs, mainly because of the inability of macrophages and dendritic cells to display Brucella antigens via major histocompatibility complex class I (MHC-I) molecules, and therefore decreasing the CD8⁺ T cell response (16). Alteration of T-cell function may be the key to explaining the clinicopathological manifestations of relapsing brucellosis. Based on these considerations, the unusual and diverse manifestations of relapsing brucellosis could be related to several potential immunopathogenic mechanisms: an ineffective CD4⁺ effector response, a downregulated CD8⁺ T cell response, or a continued, established Th2 response, each of which could result in an incomplete resolution of the infection.
A new positive blood culture. Twelve community patients in Lima who denied a history of brucellosis and who were serologically negative for Brucella infection served as negative controls. Peripheral blood mononuclear cells (PBMCs) were collected by leukopheresis (in the case of the UPCH subjects) or standard venipuncture procedures (UCSD subject), separated by Ficoll density gradient centrifugation, and cryopreserved under controlled conditions in liquid nitrogen (22).

**Antigen selection, epitope prediction, and peptide synthesis.** Fifty proteins were selected in order to study to which IgG reactivity was demonstrated on genome level protein microarray analysis (17, 18). To reduce the number of peptides to test, instead of overlapping peptides spanning all 50 proteins, HLA class II binding peptides predicted to be possible promiscuous binders from each selected open reading frame (ORF) were identified using the Immune Epitope Database and Analysis Resource (http://www.immuneepitope.org/) (23). This predictive algorithm yielded 2 to 20 peptides per protein for a total of 500 predicted potential epitopes. Peptides were synthesized (Mimotopes, Clayton, Victoria, Australia) and dissolved in dimethyl sulfoxide (DMSO) at 20 mg/ml (24).

**IFN-γ ELISPOT screening/deconvolution.** ELISPOT plates (96 wells; catalog number MAIP4510; Millipore, Billerica, MA, USA) were activated with 70% methanol, washed with sterile water, coated with mouse anti-human IFN-γ antibody (Clone 1-D1K 3420-3-1000; Mabtech, Stockholm, Sweden) and stored at 4°C overnight. Plates were washed with phosphate-buffered saline (PBS) and set up with HR-5 medium (RPMI 1640 with 5% glutamine; catalog number 11875; Invitrogen, Carlsbad, CA) with 10% penicillin-streptomycin for 1 h. Antigen pools or individual peptides derived from the pools (i.e., deconvoluted peptides) were added to HR-5 medium to a final concentration of 5 μg/ml to the ELISPOT plate in a triplicate experiment. PBMCs were removed from liquid nitrogen containment and slowly thawed in a 37°C water bath and placed into HR-5 medium with Benzonase nuclease (25 U/ml; catalog number E1014-25KU; Sigma, St. Louis, MO). PBMCs were centrifuged, washed twice with HR-5 medium, and counted for a total of 3 × 10⁵ per well in the final plate; finally, cells were centrifuged and washed prior to being added to the ELISPOT plate. Cells were incubated at 37°C and 7% CO₂ for 24 h. After incubation, cells were discarded, and ELISPOT plates were developed by washing with PBS-Tween 20 solution three times and then adding biotinylated mouse anti-human IFN-γ antibody (2 μg/ml; clone 7-B6-1; catalog number 3420-6-1000; Mabtech, Stockholm, Sweden) at 4°C for 24 h. ELISPOT plates were washed with PBS-Tween 20 solution three times, and then avidin-peroxidase complex was added (Vectastain Elite Vector PK-6100; Vector Laboratories, Burlingame, CA).

**FIG 1** Screening ELISPOT analysis. (A) Magnitude of IFN-γ ELISPOT responses to pools of *B. melitensis* antigens comparing acute brucellosis patients and negative controls. (B) Magnitude of IFN-γ ELISPOT responses to pools of *B. melitensis* antigens comparing relapsing brucellosis patients and negative controls. (C) Magnitude of IL-5 ELISPOT responses to pools of *B. melitensis* antigens comparing acute brucellosis patients and negative controls. (D) Magnitude of IL-5 ELISPOT responses to pools of *B. melitensis* antigens comparing relapsing brucellosis patients and negative controls. The data are represented as the mean of spot-forming cells (SFC) per 10⁶ ± SD.
for 1 h. Finally, plates were washed with sterile water, and an acetic acid–3-amino-9-ethylcarbazole tablet (catalog number A6926 AEC; Sigma, St. Louis, MO)/N,N-dimethylformamide (number D8654; Sigma) mixture was then activated with 30% H2O2 (number H1009; Sigma; St. Louis, MO, USA), added to wells, and rinsed with deionized water.

**Interleukin-5 screening.** For IL-5 screening, a procedure identical to that of the IFN-γ/H9253 screening was used, with the exception of the use of mouse anti-human IL-5-unconjugated antibody (number 3490-3-1000; Mabtech) as the primary coating antibody and mouse anti-human IL-5 biotinylated antibody (number 3490-6-1000; Mabtech) as the secondary antibody.

**CD4+ T cell-specific IFN-γ ELISPOT deconvolution.** The same procedure as that used for IFN-γ screening and deconvolution was used for CD4+ T cell IFN-γ deconvolution, with the exception being that the PBMCs were CD4+ T cell enriched via CD8+ T cell depletion using magnetic beads (MACS number 130-045-201; Miltenyi Biotech, Auburn, CA).

**Data acquisition and statistical analysis.** An S6 UV Core ELISPOT Reader (Cellular Technology Ltd., Cleveland, OH) was used to scan, count, and perform quality control of ELISPOT plates. Statistical analyses were performed using Student’s t test and Fisher’s exact test.

**RESULTS**

**Screen of peptide pools for Th1/Th2 cytokine production.** Twenty-seven pools encompassing 500 potential epitopes predicted from the 50 ORFs most reactive in antibody microarray data as described above were screened for reactivity with leukapheresis-derived PBMCs from 9 patients who had recovered from acute brucellosis and 4 patients who had recovered from relapsing brucellosis (plus 1 relapsed patient with PBMCs obtained from phlebotomy) (Table 1), in addition to 13 negative controls. Production of IFN-γ and IL-5 was assessed by ELISPOT (24), and the average magnitudes of responses observed for the various patient categories were determined (Fig. 1); focus was placed on the most immunogenic peptides from those patients who were positive for brucellosis. There was no difference in response rate between acute and relapsing patients (P = 0.33, Fisher’s exact test). Responses above the 100 spot-forming cells (SFCs)/million threshold were detected in 0/14 healthy controls, demonstrating that the responses were specific to Brucella-infected patients. IL-5 responses were barely detectable, and thus none reached the threshold of positivity. In conclusion, these results demonstrate that specific responses can be detected against *B. melitensis* peptides in infected individuals, but not in uninfected controls.

**Identification of *B. melitensis* epitopes.** To more precisely define the specificity of the responses detected in the experiments...
described above, positive pools were subsequently deconvoluted
(i.e., each individual peptide within a positive pool was tested).
More specifically, eight pool/patient combinations yielding re-
sponses greater than 100 SFC/million were deconvoluted for
IFN-γ/H9253 (Table 2). Using standard methodology and criteria (24), a
peptide response was considered positive if 3 of 4 separate criteria
were met, namely, for peptide pool positivity, the presence of 100
SFCs/10^6 PBMCs, a P value of ≤0.05, a stimulation index (SI) of
≥2, and a Poisson distribution of <.5.

Breadth and magnitude of responses. Next, we inspected the
ELISPOT data (Table 2) for potential differences in the breadth
and/or magnitude of responses. For the breadth of responses, the
average numbers of peptides recognized were 3.8 for acutely in-
fected individuals and 4.3 for relapsing infected individuals (P =
0.835), thus demonstrating that no significant difference in the
breadth of responses was detectable. Regarding the magnitude of
responses, the total SFC response detected was 7,222, of which
acutely infected individuals accounted for 5,192 of the 7,222,
while relapsing infected individuals accounted for 1,920 of the
7,222 (Student’s t test, P = 0.188). Taken together, these data
suggest that while there was a nonsignificant trend toward higher
and more-focused responses in relapsing versus acutely infected
individuals, no significant differences were detected between
acute-infection and relapsing patient groups.

CD4^+ T cell recognition of identified epitopes. The data pre-
presented thus far were obtained using whole, unfraccionated
PBMCs. It was expected that since the peptide epitopes identified
were selected on the basis of being promiscuous HLA class II bind-
ing peptides, CD4^+ T cells mediate their recognition. To confirm
this further, CD4^+ T cells were purified for each of the positive
responders, and then IFN-γ ELISPOT was performed using the
specific peptides that were positive for those patients. The data
(Fig. 2) demonstrated that these patient-specific CD4^+ T cells did
indeed recognize the predicted promiscuous HLA class II binding
peptides.

Dominant B. melitensis antigens and epitopes. Further analy-
sis of the data presented (Table 2) revealed that 14/50 proteins
were recognized by at least one patient and 3/50 proteins were
recognized by more than one patient (BMEI 1330, BMEII 0029,
BMEI 0691). These 3 proteins were recognized in 7/9 (78%) of
responders and in 7/13 (54%) of the total number of subjects.
These proteins produced an IFN-γ response in both acute and
relapsing patients and account for 68% of the total reactivity
noted, which demonstrates that these proteins are immunodom-
inant.

In general, each patient recognized different epitopes within
each protein, yet peptides BMEI 1330.1, BMEI 0029.3, BMEI
0029.4, and BMEI 0691.23 were seen in 7/9 (78%) of patients in
both acute-disease and relapsing groups and in 7/13 (54%) of
individuals in the total cohort.

Features of immunodominant proteins. Each of the three pro-
teins immunodominant for Th1 responses is located in a different
area of the B. melitensis ORFeome, and all three proteins have differ-
ent predicted subcellular localization and functional properties in
the bacterium (Table 3). Protein BMEI 1330, a probable Deg-P like protease, elicited high IFN-γ ELISPOT and antibody responses and is conserved across most bacteria in the Alphaproteobacteria. The other two proteins, BMEI 0029 (a VirB5-associated protein) and BMEII 0691 (a putative periplasmic binding protein), elicited a high IFN-γ response yet a low antibody response. Each of the amino acid sequences for these four epitopes is listed in Table 4.

Lack of correlation between Th1 and antibody responses. 
The data discussed above indicate a lack of correlation between recognition from Th responses and the magnitude of antibody responses directed against the same proteins. The 50 proteins analyzed for class II-restricted T cell epitopes in this study were selected on the basis of antibody reactivity. Previous studies involving viral pathogens (25) indicated that the antigens recognized by antibody responses tend to overlap and correlate with those recognized by Th responses. This observation is also in agreement with similar observations from independent studies relating to Mycobacterium tuberculosis (26).

However, prior to the present work, it was not known whether these rules could be applicable to human class II-restricted CD4+ T cell responses to B. melitensis. To examine this issue in more detail, we tested whether a correlation could be demonstrated between ranking in terms of antibody responses and immunodominance for Th responses. As noted previously, the 50 proteins used in the analysis were noted to have the highest antibody responses. Based on each protein’s intensity value, a mean was calculated, and subsequently each was placed on a spectrum depending on whether its intensity value was over or under the mean (see Table S2 in the supplemental material), and then the proteins were compared regarding whether they produced a Th1 response (Fig. 3). Only 5 proteins produced both an above-the-mean antibody response and a Th1 response. Eight proteins did not produce an above-the-mean antibody response but did produce a Th1 response. Fourteen proteins did produce an above-the-mean antibody response but did not produce a Th1 response. Twenty-three proteins produced neither an above-average antibody response nor a Th1 response (P = 0.6098; Fisher’s exact probability test).

DISCUSSION
Here, we present the first determination of human T cell recognition of peptide antigens in Brucella melitensis. The human immune responses both in recovered acute-disease and in relapsed brucellosis patients were observed to consist of only Th1, as determined by IFN-γ and IL-5 ELISPOT analysis. Potential epitopes were predicted on the basis of promiscuous binding to HLA class II molecules, to which this approach has been previously shown to be effective in predicting epitopes derived from common allergens and more recently the intracellular human pathogen Mycobacterium tuberculosis (22). Our findings highlight that the approach of predicting promiscuous T cell epitopes is also applicable to B. melitensis and thus argue in favor of its general applicability.

In terms of the quality of responses, we were able to readily detect Th1 responses but not Th2 responses. This is consistent with what is observed in the case of other intracellular bacteria, where cells of the Th1 subset mediate the predominant response (27, 28). This conclusion should be interpreted with caution, since these analyses are based on single leukapheresed samples derived from a 2005 to 2009 epidemic in Peru, and accordingly, we have been unable to perform a longitudinal analysis of PBMC reactivity. The low number of cells available to us limited the number of cytokines that we were able to measure. IL-5 was taken as a representative Th2 lymphokine, as it is well established (29, 30) that this lymphokine is most readily detected by the ELISPOT assay, compared to other Th2 lymphokines (e.g., IL-4 and IL-13). Future studies will examine the possibility that other Th subsets such as Treg and Th17 may be involved in responses.

Our findings indicate that epitope-specific IFN-γ responses can be used to discriminate B. melitensis-infected individuals from uninfected controls. In addition to being the first identification of human B. melitensis epitopes, the current report demonstrates the potential practical diagnostic use of these epitopes. Three proteins (with a total of four epitopes) generated a significant Th1 response

![FIG 3](http://iai.asm.org/) Flow diagram categorizing the breadth of the antibody (Ab) response to the top 50 B. melitensis proteins and their Th1 responsiveness. Patient antibody responses to the top 50 B. melitensis proteins (17) were separated into whether they were above or below the mean of the intensity (SD ± 12,000) to their respective Th1 responses. See Table S2 in the supplemental material for a display of the numerical values of the intensity of the antibody response to the top 50 B. melitensis proteins.

**TABLE 3** The three B. melitensis immunodominant antigens associated with positive IFN-γ responses

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cellular location</th>
<th>Function</th>
<th>Reference sequence accession no.</th>
<th>Superfamily</th>
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<tbody>
<tr>
<td>BMEI 1330</td>
<td>Periplasm</td>
<td>Seryl-tRNA synthetase</td>
<td>NP_539999.2</td>
<td>DegP/HtrA protease</td>
</tr>
<tr>
<td>BMEI 0029</td>
<td>Extracellular membrane</td>
<td>Type 4 secretion system</td>
<td>NP_541006</td>
<td>VirB5 protein</td>
</tr>
<tr>
<td>BMEII 0691</td>
<td>Periplasm</td>
<td>Unknown</td>
<td>NP_541669.1</td>
<td>Putative oligopeptide transport system</td>
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</table>

**TABLE 4** Complete peptide sequence for each of the four B. melitensis immunodominant T cell epitopes

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide sequence</th>
</tr>
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<tbody>
<tr>
<td>BMEI 1330.1</td>
<td>SNTRKGVAAVALSAA</td>
</tr>
<tr>
<td>BMEI 0029.3</td>
<td>TDKRLQIEISLMQE1</td>
</tr>
<tr>
<td>BMEI 0029.4</td>
<td>DTRLHLYQQMAEQD</td>
</tr>
<tr>
<td>BMEII 0691.23</td>
<td>DTYVPVLYHLPYQWA</td>
</tr>
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</table>

*Each of these sequences is a peptide sequence derived from each of the complete proteins listed in Table 3.*
in more than one of those individuals from either cohort, accounting for 68% of the total IFN-γ responses, and are thus by definition immunodominant, at least within the 50 protein targets tested. This observation is of considerable practical potential relevance, as illustrated by the fact that an assay based on the detection of IFN-γ in response to pathogen-deriv peptide is the gold standard diagnostic assay utilized in the case of Mycobacterium tuberculosis (Quantiferon Gold) (27). The Quantiferon Gold and similar assays do not allow for the discrimination of active versus chronic tuberculosis. In our investigation, there was no significant difference demonstrated in the Th1 responses to B. melitensis epitopes between acute and chronic/relapsing brucellosis patients in the breadth, magnitude, or specificities of these Th1 responses.

Recent observations have suggested that a defined, epitope-specific Th1 response is necessary for control of brucellosis (31), yet it was crucial to discover any possible overlap between immunodominant Th1 epitopes and the antibody response against B. melitensis antigens. This was the central point of the investigation and had not previously been undertaken for human brucellosis. Earlier studies involving other intracellular pathogens (such as M. tuberculosis (26)) have indicated that the antigens recognized by antibody responses tend to overlap and correlate with those recognized by Th responses. The 50 proteins of B. melitensis analyzed herein were selected on the basis of high antibody reactivity from Peruvian brucellosis patients; the results presented indicate that 1 of these 50 antibody-associated immunogenic B. melitensis proteins also had a Th1 response (BMEI 1330). This is also in agreement with similar observations from independent studies relating to M. tuberculosis (32, 33). This finding has profound implications, as it suggests that the current study might have just scratched the surface of the potential definition of targets for T cell recognition in B. melitensis.

Our investigation identified 3 immunodominant proteins of 50 tested. BMEI 1330 is a Seryl-tRNA synthetase that is also found in other Brucella spp. BMEI 0029/VirB5, part of the type IV secretion system, is essential for the interaction of Brucella spp. with host cells and importantly, survival within macrophages (34). BMEI 0691 is a putative, periplasmic oligopeptide transporter, and is part of the OppA superfamily; its structure is similar to the same protein found in other Brucella spp. and in Ochrobactrum anthropi. Interestingly, neither BMEI 0536, a well-known immunodominant 26-kDa protein (as determined by antibody response), nor BMEI 0402, a 31-kDa outer membrane protein (6, 35–37), generated a significant IFN-γ response by ELISPOT analysis.

One of the limitations of this investigation was that HLA typing was not performed on these samples. This was due to the study design of the computational prediction of these T cell epitopes, which would be independent of the individual’s HLA type. In addition, CD8+ T cell responses were not measured, as the study design protocol was geared for MHC-II promiscuous T cell epitopes only. Considering that several thousand ORFs remain to be examined, extrapolating from the current hit rate, it is possible to speculate that tens if not hundreds of different antigens remain to be discovered. These data thus provide a compelling rationale for a T cell-based genomic screen of B. melitensis.

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REFERENCES

B. melitensis Epitope Identification


