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Discordant *Brucella melitensis* Antigens Yield Cognate CD8$^+$ T Cells In Vivo$^\nabla$

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***Brucella* spp. are intracellular bacteria that cause the most frequent zoonosis in the world. Although recent work has advanced the field of *Brucella* vaccine development, there remains no safe human vaccine. In order to produce a safe and effective human vaccine, the immune response to *Brucella* spp. requires greater understanding. Induction of *Brucella*-specific CD8$^+$ T cells is considered an important aspect of the host response; however, the CD8$^+$ T-cell response is not clearly defined. Discovering the epitope containing antigens recognized by *Brucella*-specific CD8$^+$ T cells and correlating them with microarray data will aid in determining proteins critical for vaccine development that cover a kinetic continuum during infection. Developing tools to take advantage of the BALB/c mouse model of *Brucella melitensis* infection will help to clarify the correlates of immunity and improve the efficacy of this model. Two H-2^d CD8$^+$ T-cell epitopes have been characterized, and a group of immunogenic proteins have provoked gamma interferon production by CD8$^+$ T cells. RYCINSASL and NGSSSMATV induced cognate CD8$^+$ T cells after peptide immunization that showed specific killing in vivo. Importantly, we found by microarray analysis that the genes encoding these epitopes are differentially expressed following macrophage infection, further emphasizing that these discordant genes may play an important role in the pathogenesis of *B. melitensis* infection.**

Brucellosis is the world's most common zoonosis, with more than half a million new human infections each year (44). Brucellosis has been endemic to the Mediterranean and Middle East since ancient times, since carbonized cheese and skeletal remains in Pompeii show evidence of *Brucella* spp. (8). Evidence of brucellosis also exists in the skeleton of a 2.4- to 2.8-million-year-old hominid (16). In areas of endemicity, domestic animal brucellosis severely affects regional economies, and vaccination campaigns cannot always reach nomadic herders. Human infections occur in these regions mainly from the ingestion of infected animal products, including unpasteurized milk and fresh cheeses (14). Antibiotic treatment exists but is costly and prolonged, lasting at least 6 weeks in moderate cases, and it may extend for years depending on complications that arise. Even after treatment, PCR data have revealed that low levels of bacteria are detectable years after the resolution of symptoms, and relapses occur in 5 to 30% of cases (20, 30, 55, 62). In areas where brucellosis is endemic, prevention of infection via vaccine would be far more cost-effective than the regimen of antibiotics suggested by the World Health Organization (WHO). Unfortunately, this disease flies below the radar of many of the major world health agencies, and the problem is compounded by frequent misdiagnosis and under-reporting (15, 20).

Although brucellosis is eradicated from food sources here, in the post-Gulf War United States, awareness was raised to fund vaccine research concerning potential biological weapons. *Brucella melitensis*, *B. abortus*, and *B. suis* are considered category B select agents because of the ease of aerosolization, diverse symptoms, and chronic persistence. The spectrum of disease that results from *Brucella* infection suggests that *Brucella* spp. could be a biological weapon in the current absence of any human vaccine (43). Human symptoms begin with a general malaise and fever, followed by organ-specific "hot spots" of infection, for instance, endocarditis and orchitis. In the United States, infections are due to accidental infection with a live animal vaccine by veterinarians and laboratory workers. In fact, brucellosis is one of the most common laboratory-acquired infections, and the lack of a human vaccine discourages work with the agent (20, 37, 40).

Three vaccines are currently recommended by the WHO for livestock, and all of them are live-attenuated *Brucella* strains: *B. abortus* S-19 and RB-51 for bovine brucellosis and *B. melitensis* Rev-1 for goat and sheep brucellosis. These vaccine constructs are not completely effective and pose safety risks, including abortifacient effects and residual virulence, making them unsuitable for human application (33). Heat-killed *Brucella* does not induce detectable interleukin-12 (IL-12) in vivo, and killed bacteria actively suppress IL-12 production in response to challenge with live bacteria by unknown mechanisms (24). Studies conducted in our laboratory, and confirmed by others, have shown that subunit vaccines can confer a degree of short-term protection but have not elicited long-term effective immunity (3, 39). Only live bacteria appear to induce cell-mediated immunity, whereas dead bacteria induce a nonprotective humoral response (31, 36).

CD4$^+$ T cells induce the production of IgG2 antibodies from B cells during the course of murine and ovine *B. melitensis* infections (9, 56). There is evidence that this humoral response

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is an indispensable aspect of the host defenses in that opsonization may be required for successful uptake by macrophages, although a humoral response is not protective (7, 18, 31). In addition, although opsonization may result in increased bacterial uptake by macrophages, bacterial survival is unchanged (18). Previous studies have shown that host protection can be mediated by gamma interferon (IFN-γ) produced by $CD4^+$ T cells, although data have also shown that treatment of macrophages with optimal concentrations of IFN-γ still allows some intracellular *Brucella* to survive (19, 26, 57, 63). *Brucella* can escape complement-mediated killing and thrive inside the acidified phagosomes of macrophages, using the common bactericidal host mechanisms to its own advantage (11, 13, 28a). In addition, major histocompatibility complex (MHC) class II antigen presentation can be disrupted by *Brucella* lipopolysaccharide that has incorporated into the host cell membrane (28). In our lab and others, evidence supports that protection in animal models is engendered by $CD8^+$ T cells (10, 12, 22, 27, 38, 42, 64). Therefore, we chose to investigate the *Brucella* antigens that are recognized by $CD8^+$ T cells in the context of MHC class I molecules.

In the United States, most select agent work is confined to biosafety level 3 and above, the logistics of which largely dictate the use of small-animal models in *Brucella* research. Mice are not a natural host of *B. melitensis*, making the optimization of this model a high priority. By exploring the $CD8^+$ T-cell component of the BALB/c mouse response to *B. melitensis* infection, we are further refining the mouse as a valuable tool in *Brucella* research and vaccine development.

Determining the epitopes recognized by *Brucella*-specific $CD8^+$ T cells and the *Brucella* genes encoding the proteins containing these epitopes will help establish proteins critical for vaccine development (47, 48, 51, 52, 60). Epitopes were predicted from the *Brucella* genome using an algorithm based on allele-specific binding motifs and cleavage sites (49, 50). Select peptides were then tested for their capacity to bind their respective MHC alleles in vitro (54). Peptides subsequently deemed epitopes displayed a combination of immunogenicity, natural processing, and functional avidity, while eliciting $CD8^+$ T cells that kill in vivo. Peptide immunogenicity was evaluated using peptide pools in adjuvant, whereas natural processing and functional avidity tests used nonreplicating but metabolically active whole *B. melitensis* to immunize mice. Our approach has identified the first *B. melitensis*-specific MHC class I $CD8^+$ T-cell epitopes that are recognized in $H\text{-}2^d$ mice and generate $CD8^+$ T cells that kill in vivo. These present findings offer insight regarding the debate concerning *Brucella* correlates of immunity and provide guidance in designing a safe and viable human vaccine.

MATERIALS AND METHODS

Peptide prediction and synthesis. The *B. melitensis* strain 16M open reading frames utilized in the present study correspond to NCBI accession numbers NC_003317 and NC_003318. Candidate $H\text{-}2^d$ epitopes were identified by using RankPep (http://immunax.dfci.harvard.edu/Tools/rankpep.html), a previously described algorithm (49, 50). Further peptide prediction utilized the Immune Epitope Database (IEDB) stabilized matrix method algorithm at http://tools.immuneepitope.org (45). 8- and 9-mer MHC class I peptides (≥90% pure) were obtained from Synthetic Biomolecules, Inc. (San Diego, CA).

$H\text{-}2K^d$ and $H\text{-}2D^d$ peptide-binding assay. The binding of peptides to purified K^d and D^d molecules was quantified in competition assays based on the inhibition of binding of a high-affinity radiolabeled standard probe peptide as previously described (41, 54). Briefly, competitor peptides were coincubated for 48 h at room temperature with purified MHC, a high-affinity radiolabeled probe peptide, and β_2-microglobulin in the presence of a cocktail of protease inhibitor. Peptides were tested at six different concentrations covering a 100,000-fold dose range in three or more independent assays. After a 2-day incubation, MHC-peptide complexes were captured on Greiner Lumitrac 600 microplates (Greiner Bio-One, Monroe, NC) coated with either anti-$H\text{-}2K^d$ SF1.1.1 or anti-$H\text{-}2D^d$ 34-5-8s antibody, and binding was determined by measuring the counts per minute (cpm) using a Topcount microscintillation counter (Packard Instruments, Waltham, MA). For each peptide, the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC_{50}) was calculated. Under the conditions used, where [radiolabeled probe] < [MHC] and IC_{50} > [MHC], the measured IC_{50} values are reasonable approximations of the true K_d values. Peptides with an affinity of 500 nM or greater were considered binders, reflecting a threshold previously shown to be associated with T-cell recognition in vivo in murine, human, and rhesus systems (4, 53, 61).

Immunization of mice. Female BALB/c mice were obtained from Harlan (Indianapolis, IN) at 6 to 8 weeks of age and housed in AAALAC-approved facilities under pathogen-free conditions using standard protocols. For immunogenicity, functional avidity and in vivo killing studies, groups of four mice at 6 to 8 weeks of age were immunized subcutaneously at the base of the tail with 50 μg of each peptide in phosphate-buffered saline (PBS)–10% dimethyl sulfoxide emulsified 1:1 in incomplete Freund adjuvant (IFA) or in IFA alone. For the antigen processing studies, groups of 12 mice were immunized intraperitoneally (i.p.) with 10^8 replication-deficient, but metabolically active *B. melitensis*-green fluorescent protein (GFP) in 0.1 ml of PBS previously irradiated with 350 kilorads as described previously (29). Briefly, log phase bacteria were collected, pelleted, and resuspended in fresh brucella broth. Cultures were then irradiated at 350 kilorads using a $cesium^{137}$ Mark I irradiator (J. L. Shepard Co., San Fernando, CA). Lack of replication was confirmed by assaying growth on brucella agar after 72 h at 37°C. GFP-expressing *B. melitensis* was used wherever possible so that the responses of defined class I GFP epitope-specific T cells were used for the positive control and assay verification. All mouse experiments reported herein were repeated three or more times. Experiments with *Brucella* cultures and infected cells were done according to protocols approved by the Institutional Biosafety Committee and Biological Safety Office.

Intracellular cytokine assay. Splenocytes from experimental and control mice were passed through a 70-μm-pore-size strainer and treated with ACK buffer (Quality Biologicals, Gaithersburg, MD). Cells were then cultured in 96-well round-bottom plates (10^6 cells/well) in complete medium in the presence of 100 μg to 0.1 ng of purified MHC class I peptide/ml and 10 μg of GolgiPlug (BD Biosciences, San Jose, CA)/ml, with or without concanavalin A (Sigma-Aldrich, St. Louis, MO). After 5 h, the cells were surface stained with phycoerythrin-conjugated anti-mouse CD4 and PerCP anti-mouse CD8 and then fixed and permeabilized according to the Cytofix/Cytoperm manufacturer's protocol, followed by intracellular staining with fluorescein isothiocyanate-labeled anti-mouse IFN-γ (BD Biosciences). Flow cytometry for the immunogenicity and antigen processing studies was performed on a FACScan (BD Biosciences). Functional avidity and in vivo killing studies were analyzed by using an FC500 (Beckman Coulter, Fullerton, CA). The data were further analyzed by using FlowJo software (Tree Star, Ashland, OR).

In vivo killing assay. Splenocytes from donor BALB/c mice were labeled with 5.0- or 0.5-μm carboxyfluorescein diacetate succinimidyl ester (CFSE; high and low concentrations, respectively). $CFSE^{lo}$ cells were pulsed with 1 μg of irrelevant peptide (GYKVAPAAL)/ml, and $CFSE^{hi}$ cells were pulsed with 1 μg of NGSSSMATV or RYCINSASL/ml. Equal amounts of $CFSE^{hi}$ and $CFSE^{lo}$ were combined and transferred (~10^7 total cells/mouse) via intraorbital injection into syngeneic mice that had been peptide immunized 7 days prior. After 6 h, CFSE-labeled cells were recovered from whole splenocytes and analyzed by flow cytometry. The percent killing was calculated as [1 − (the ratio of irrelevant to epitope-specific cells in naive mice/the ratio in immunized mice) × 100 (25).

Statistical analysis. To determine statistical significance in the peptide assays, analysis of variance (ANOVA) was performed on data against the no-peptide control. A P value of ≤0.05 was considered significant. The data were normalized by subtracting the percentage of cells that scored positive for IFN-γ production in the absence of peptide.

Microarray. Microarray analysis was performed using *Brucella* RNA isolated from RAW 264.7 (ATCC TIB71) cells infected at a multiplicity of infection of 100 for 22 h or bacteria grown to log phase in brucella broth. Briefly, cells were suspended in Bacterial Protect reagent (Qiagen, Valencia, CA), disrupted in lysis buffer (Tris-EDTA containing 66 μg of proteinase K

TABLE 1. Summary of *B. melitensis* peptide screening results

B. melitensis ORF[a]	*B. melitensis* peptide sequence[b]	Cellular localization	*P* value[c]		Functional avidity[f]
			Immunogenicity[d]	Processing from BALB/c APCs[e]	
BMEII1097	NGPASSTTL	Unknown	<0.05		
BMEII1097	VFSEIATSV	Unknown			
BMEII0819	KYQKSAEAI	Cytoplasm			
BMEII0819	RYCINSASL	Cytoplasm	<0.05	<0.05	Yes
BMEII0699	AYASIPALL	Cytoplasm	<0.01		
BMEII0699	AGGAAYASI	Cytoplasm			
BMEII0561	GYAKMTSDL	Cytoplasm			
BMEII0561	AYLAVSEAL	Cytoplasm	<0.01		
BMEII0405	SYSEIARAI	Cytoplasm	<0.01		
BMEII0405	AFRSAFVRI	Cytoplasm		<0.001	
BMEI2035	AYQEIVKAL	Cytoplasmic membrane			
BMEI2035	IYDRYANKL	Cytoplasmic membrane			
BMEI1981	AYQPALEKI	Cytoplasm		<0.01	
BMEI1981	SGGAARLAI	Cytoplasm			
BMEI1961	SFQPVIDAI	Cytoplasm			
BMEI1961	NGSSSMATV	Cytoplasm	<0.01	<0.001	Yes
BMEI1916	TYRAVAKAL	Cytoplasm	<0.001		
BMEI1916	LFVTASPEV	Cytoplasm			
BMEI1862	NYHITLRFI	Unknown	<0.05		
BMEI1862	SGRANFATL	Unknown	<0.05		
BMEI1809	FYTASYSSV	Unknown			
BMEI1770	AGPKLIAAL	Cytoplasm			
BMEI1770	SPNRAAATL	Cytoplasm	<0.01		
BMEI1570	VFSLVVSDI	Cytoplasm	<0.05		
BMEI1570	SGGETTVTI	Cytoplasm			
BMEI1522	MYAAMAKAL	Cytoplasm			
BMEI1522	AREAVMAFL	Cytoplasm			
BMEI0485	LYEAAREAL	Cytoplasm			
BMEI0485	AYAKRAAEL	Cytoplasm	<0.01		
BMEI0445	FYALRGLSL	Cytoplasmic membrane			
BMEI0344	KGQASRAVI	Cytoplasmic membrane			
BMEI0344	GYKVAPAAL	Cytoplasmic membrane	<0.01		
BMEI0196	AYREMTGKI	Unknown	<0.05		
BMEI0196	AYTSVAEML	Unknown			
BMEI0160	SYAEVRAAL	Cytoplasmic membrane	<0.01		
BMEI0160	TFFTVVVGL	Cytoplasmic membrane			
BMEI0147	ARNAAVLTL	Unknown			
BMEI0147	AYERDTRQF	Unknown			
BMEI0001	VPLSFAAL	Cytoplasm			
BMEI0001	LEPVYETV	Cytoplasm			

[a] Peptide position within the *Brucella* genome is given as an open reading frame (ORF) designation.
[b] The peptide sequences of the predicted *Brucella*-specific epitopes from within the proteins are shown with their cellular localization (column 3).
[c] Results are based on the combined data from all peptides for immunogenicity and natural processing studies; significance was determined by ANOVA.
[d] Splenocytes were isolated from peptide immunized BALB/c mice 10 days after immunization. We looked for the $CD8^+$ T-cell IFN-γ response to peptide pulsed target splenocytes (Fig. 1).
[e] Each peptide was evaluated for its ability to elicit IFN-γ production from $CD8^+$ T cells generated from BALB/c mice immunized with metabolically active but replication-deficient *B. melitensis* (Fig. 2) whose naturally processed antigens were presented in vivo. APCs, antigen-presenting cells.
[f] The peptides that showed significant evidence of both immunogenicity and natural processing were tested for functional avidity. Splenocytes from peptide immunized BALB/c mice were isolated and pulsed with decreasing concentrations of peptide. Both epitopes were able to elicit IFN-γ from effector $CD8^+$ T cells down to a concentration of 1 ng/ml (Fig. 3).

[Epicentre, Madison, WI]/ml) and 0.193 kilounits (KU) of ReadyLyse (Epicentre)/ml, and then isolated in RLT buffer with β-mercaptoethanol (Qiagen). Samples were transferred to tubes containing 30- to 50-mg acid-washed glass beads (1.0 mm) and mechanically disrupted in a BeadBeater (Biospec Products, Inc., Bartlesville, OK). RNA was isolated by using an RNeasy kit (Qiagen) according to the manufacturer's protocol. On-column DNase treatment was included in the isolation protocol. Enrichment of bacterial RNA in RAW 264.7 cells was done by using a MicroEnrich kit (Ambion, Austin, TX) according to the manufacturer's protocol. Double-stranded cDNA was synthesized by using an Invitrogen Superscript II kit, substituting genome-directed primers (GDPs) (58). After cDNA synthesis, a clean-up step of phenol-chloroform-isoamyl alcohol was performed, and DNA was precipitated using ammonium acetate. The yield and quality of RNA and cDNA were determined by using a Nanodrop spectrophotometer and an Agilent bioanalyzer. Samples determined acceptable for microarray hybridization were labeled and hybridized utilizing a Roche NimbleGen protocol. Labeled samples (1.5 μg) were hybridized to *B. melitensis* (Roche NimbleGen A4357-001-01) for 18 to 20 h at 42°C. The slides were washed according to the manufacturer's protocol and scanned at 5 μm at a wavelength of 532 nm using a GenePix 4000B scanner (Molecular Devices Corp., Sunnyvale, CA). cDNA synthesized from RNA isolated from uninfected RAW 264.7 cells using GDP was used as a negative control. No signal was detected on the microarray. Roche NimbleGen software was used to determine fluorescence intensity levels and for quantile normalization. A robust multichip average (RMA) algorithm was used to generate gene expression signals. The EBArrays package in R was used to identify significantly changed genes (posterior probability for differential ≥ 0.50). Genome annotations and classification of proteins by clusters of orthologous groups (COG) of proteins were obtained from the RefSeq database at the National Center for Biotechnology Information and the PathoSystems Resource Integration Center (PATRIC) (46, 59).

RESULTS

Identification of CD8$^+$ T-cell epitope candidates and MHC binding. To identify candidate CD8$^+$ T-cell epitopes from *B. melitensis*, the entire genome was probed for potential H-2K^d and H-2D^d MHC class I epitopes (49, 50). We first used an algorithm, RankPep (http://immunax.dfci.harvard.edu/Tools/rankpep.html), to predict CD8$^+$ T-cell epitopes that could bind either H-2K^d or H-2D^d and verified our predictions using the IEDB T-cell prediction resource (http://tools.immuneepitope.org). The prediction is based predominantly on the binding motif of the MHC allele and the presence of possible C terminus cleavage sites (49, 50). Our analysis predicted 6,029 possible 8- and 9-mer epitopes from the *B. melitensis* genome (data not shown). To begin experiments with a manageable set of peptides and proteins, 18 peptides possessing top RankPep scores were chosen. The next-highest scored peptide was taken from the same protein to keep our protein pool of manageable size. Finally, 4 peptides that had very low RankPep scores were included as controls, to give a total group of 40 *B. melitensis* peptides, 30 predicted to bind H-2K^d and 10 predicted to bind H-2D^d (Table 1). To confirm which of the *B. melitensis* specific peptides would functionally bind to BALB/c mouse MHC class I H-2K^d and H-2D^d alleles, binding affinity (IC50 nM) was measured in competitive inhibition assays using purified MHC molecules. As shown in Table 2, 25 peptides bound H-2K^d, and 4 peptides bound H-2D^d with an affinity of 500 nM or greater.

Immunogenicity of predicted *B. melitensis* specific MHC class I epitopes. Next, we investigated the ability of the *B. melitensis*-specific peptides to elicit an immunogenic response by immunizing BALB/c mice with pools of peptides, including peptides that did not bind MHC alleles in vitro. Purified peptides were randomly pooled into groups of 10 with 50 μg of each peptide included in the preparation with IFA for subcutaneous injection. After 8 to 10 days, the splenocytes were isolated and pulsed with individual peptides ex vivo, and specific CD8$^+$ T-cell activation was assessed by quantifying IFN-γ production (Fig. 1). We observed that 14 of the H-2K^d and 1 of the H-2D^d binding peptides were recognized in vitro (Table 1).

Natural processing of predicted *B. melitensis*-specific epitopes from native antigen. To determine whether any of the predicted peptides would be presented by MHC class I after host infection with intact *B. melitensis*, mice were injected with 10^8 metabolically active, irradiated *B. melitensis*-GFP. Eight to 10 days later, splenocytes were isolated for intracellular cytokine staining (Fig. 2A). Isolated splenocytes were then pulsed with the individual purified *B. melitensis* peptides or class I GFP epitope as the positive control. After mouse immunization with whole *Brucella*, lymphocyte activation after a peptide pulse results from bacteria being naturally processed by the antigen-presenting cells, with presentation of *B. melitensis* peptide to responding CD8$^+$ T cells. IFN-γ production by activated splenic CD8$^+$ T cells was indicative of natural processing and presentation of the predicted peptide. As shown in Fig. 2B, four predicted peptides displayed evidence of natural processing, and two of these were also immunogenic (Table 1).

Avidity of CD8$^+$ T-cell responses against *B. melitensis* peptides. We also sought to determine whether any of the probable epitopes could activate specific CD8$^+$ T cells at physiologically relevant concentrations (Fig. 3). Mice were injected with 10^8 metabolically active, irradiated *B. melitensis*, and 9 days later splenocytes were harvested. Next, splenocytes were incubated with serially diluted purified peptide, and IFN-γ production was used as an indicator of CD8$^+$ T-cell activation. Two peptides that demonstrated both immunogenicity and natural processing, RYCINSASL and NGSSSMATV, also activated CD8$^+$ T cells at physiologically relevant concentrations (Table 1). One other *Brucella*-specific peptide, AYQPALEKI, was not immunogenic in the peptide pool but did show significant evidence of natural processing and was also able to activate CD8$^+$ T cells in these experiments at a level in between the other two epitopes (data not shown).

Peptide immunization of BALB/c mice induces CD8$^+$ T cells that specifically kill in vivo. By functional assay, we evaluated the ability of these newly identified epitopes to generate CD8$^+$ T cells that kill in vivo. At 7 days after peptide immunization, recipient mice received donor cells that had been pulsed with the epitopes or irrelevant peptide and stained with CFSE^{hi} (epitope specific) or CFSE^{lo} (irrelevant). Two of the *B. melitensis* peptides were able to induce specific killing as shown in Fig. 4. Immunization with NGSSSMATV resulted in the highest level of specific killing, ranging from 33 to 68%. RYC

TABLE 2. In vitro binding of peptides to MHC[a]

Binding affinity and *B. melitensis* ORF	*B. melitensis* peptide sequence	H-2K^d or H-2D^d binding affinity (IC_{50} [nM])[b]
H-2K^d binding affinity		
BMEI1809	FYTASYSSV	0.1
BMEI1981	AYQPALEKI	0.1
BMEI1961	SFQPVIDAI	0.1
BMEI0160	SYAEVRAAL	0.2
BMEII0405	SYSEIARAI	0.2
BMEII0561	GYAKMTSDL	0.4
BMEI1862	NYHITLRFI	0.6
BMEII0561	AYLAVSEAL	1.2
BMEII0819	KYQKSAEAI	1.3
BMEI0196	AYREMTGKI	2.3
BMEI0485	LYEAAREAL	10.1
BMEI0445	FYALRGLSL	15.4
BMEI1916	TYRAVAKAL	25.9
BMEI0485	AYAKRAAEL	26.3
BMEI1522	MYAAMAKAL	30.1
BMEII0819	RYCINSASL	45.5
BMEII1097	NGPASSTTL	60.1
BMEI1770	AGPKLIAAL	67.1
BMEI1570	VFSLVVSDI	75.6
BMEII0699	AYASIPALL	139.8
BMEII0699	AGGAAYASI	201.4
BMEI0001	VPLSFAAL	247.8
BMEI1522	AREAVMAFL	248.0
BMEI2035	AYQEIVKAL	309.7
BMEI0344	KGQASRAVI	331.5
H-2D^d binding affinity		
BMEI1770	AGPKLIAAL	13.4
BMEI0445	FYALRGLSL	61.7
BMEII1097	NGPASSTTL	222.5
BMEI1961	SFQPVIDAI	323.8

[a] That is, synthesized peptide binding to H-2K^d and H-2D^d, as indicated in column 1.

[b] High and intermediate binding affinities are indicated by IC_{50}s of <50 and <500 nM, respectively.

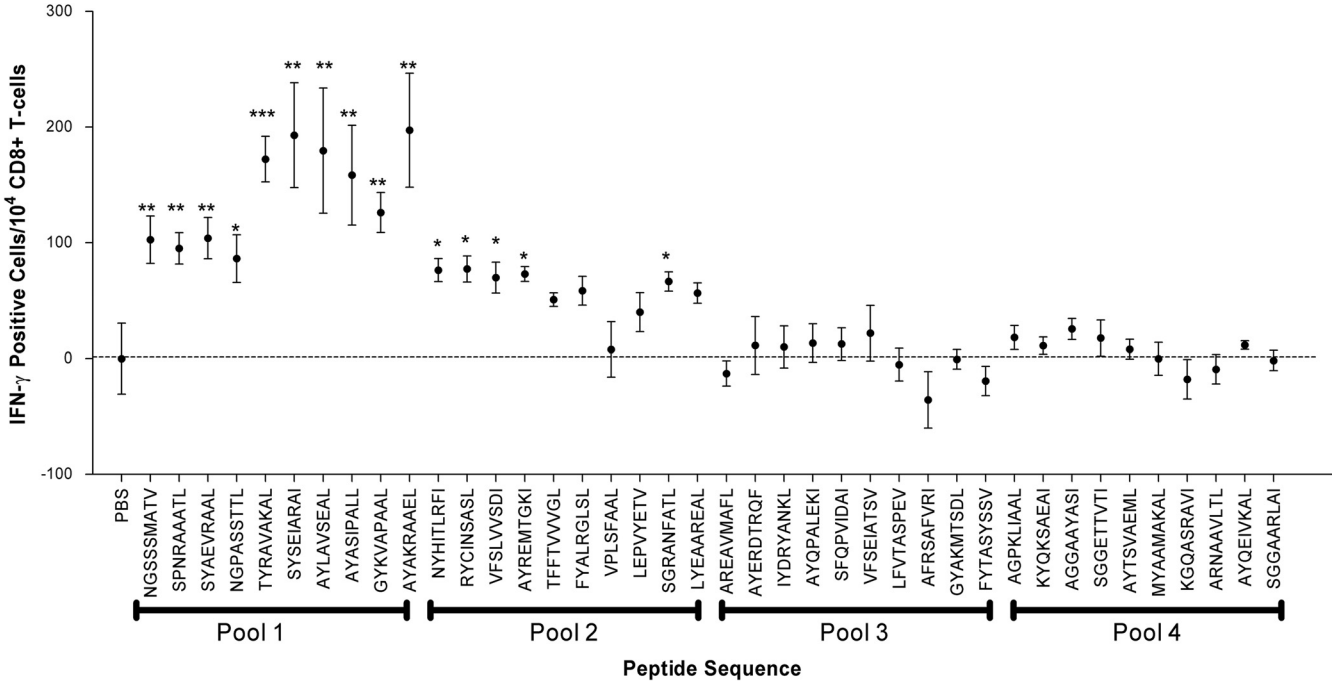

FIG. 1. Immunogenicity of *B. melitensis* peptides. BALB/c mice were vaccinated with pools of 10 peptides, 50 μg each, by subcutaneous injection at the base of the tail. The frequency of $CD8^+$ IFN-γ^+ T cells after peptide pulse in the presence of brefeldin A was determined by intracellular cytokine staining. Three replicates were performed, and significance determined by ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

INSASL showed detectable killing as well, although lower than NGSSSMATV (range, 25 to 46%).

Differential expression of epitope-containing proteins following macrophage infection. Murine RAW 264.7 (H-2^d) macrophages were infected with *B. melitensis* for 22 h, and bacterial RNA was then isolated. Interestingly, genes that contain 2 of the *Brucella* epitopes, BMEII 0819 and BMEI 1961, significantly changed in expression levels following intracellular infection (Fig. 5). Transcription of BMEII 0819, a transcriptional regulator containing the RYCINSASL epitope, was significantly downregulated after macrophage infection with a $\log_2$ ratio of −1.31. Transcription of BMEI 1961, a polyribonucleotide nucleotidyltransferase containing the NGSSSMATV epitope, was significantly upregulated after macrophage infection with a $\log_2$ ratio of 1.09. The data are drawn from one experimental condition after 22 h of infection. Further studies will determine the significance of these changes, although we propose that these data will be important in producing a mul-

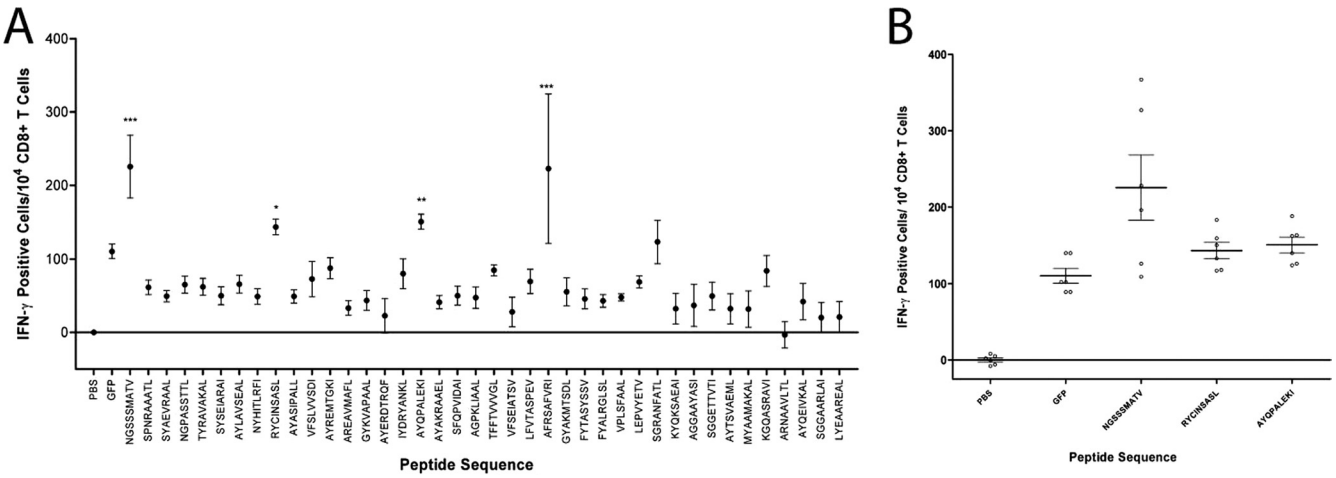

FIG. 2. Natural processing of *B. melitensis* peptides after infection with irradiated *B. melitensis*-GFP. BALB/c mice were inoculated with irradiated *B. melitensis* via i.p. injection. Splenocytes were isolated 9 days later and pulsed with purified peptide in the presence of brefeldin A. Pulse results obtained with a known class I GFP peptide are included as a positive control. (A) Combined data from all peptides. Significance was determined by ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Data from positive peptides show experimental replicates.

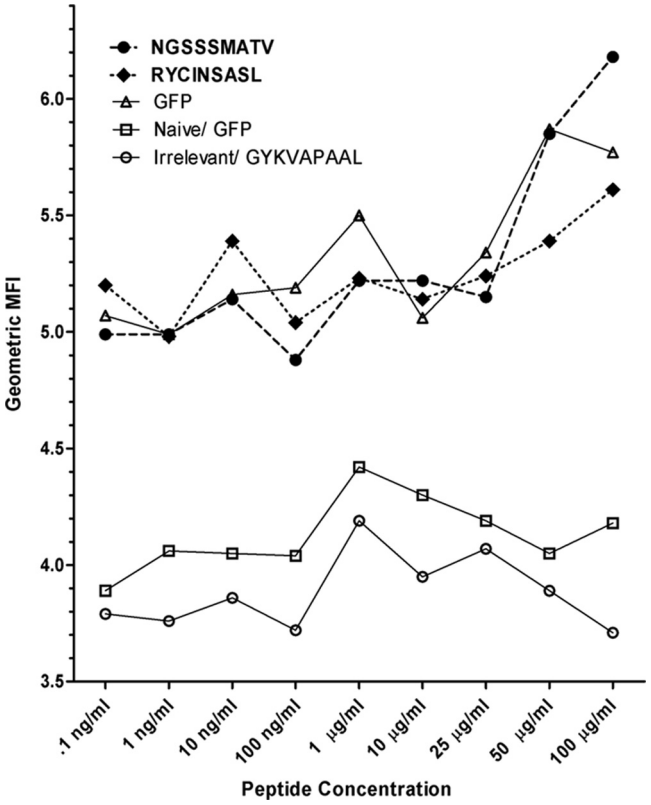

FIG. 3. Functional avidity of select peptides. BALB/c mice were inoculated with inactivated *B. melitensis* via i.p. injection. Splenocytes were isolated 9 days later and pulsed with gradient doses of purified peptide in the presence of brefeldin A. The mean fluorescence intensity (MFI) of IFN-γ staining in $CD8^+$ T cells ($n = 6$) was determined. The data from three independent experiments are shown.

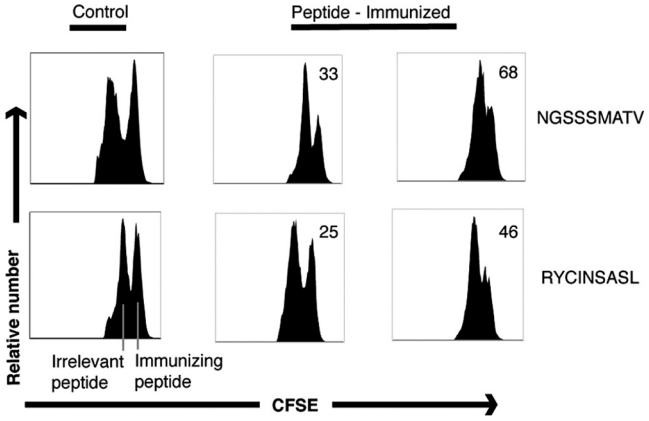

FIG. 4. In vivo specific killing. In vivo killing of target cells that had been pulsed with peptide in BALB/c mice after peptide immunization was evaluated. H-$2K^d$ mice were immunized with NGSSSMATV, RY CINSASL, or adjuvant alone. After 7 days, CFSE-labeled target cells ($CFSE^{lo}$ pulsed with irrelevant peptide or $CFSE^{hi}$ pulsed with epitope-specific peptide) were transferred to recipient syngeneic mice via intraorbital injection. After 6 h, the CFSE-labeled cells were recovered and enumerated. Numbers represent the percent specific killing.

tivalent vaccine containing antigens that cover the continuum of *B. melitensis* infection, combining immunogenic epitopes from different phases of infection. Also, in order to elucidate the spectrum of up- and downregulation of these particular transcripts, additional microarrays coupled with quantitative reverse transcription-PCR (qRT-PCR) will be necessary to cover a variety of time points and host cell types.

DISCUSSION

The importance of inducing cellular immunity to intracellular pathogens is well established (32, 47, 48, 51, 52, 60). Extensive research into host mechanisms of protection against *Brucella* spp. have shown that whereas both $CD4^+$ T cells and $CD8^+$ T cells are involved in the host response to *Brucella* infection, $CD8^+$ T cells are particularly crucial. Mice lacking β_2-microglobulin, which cannot make a functional $CD8^+$ T-cell response, have significantly exacerbated brucellosis (38). Studies using in vivo depletion of T-cell subsets have shown that $CD8^+$ T cells are the primary responder to DNA vaccines encoding a *B. melitensis* and *B. ovis* outer membrane protein (10). In addition, in vivo depletion of $CD8^+$ T cells results in higher bacterial load in *B. abortus*-infected BALB/c mice (35). Although $CD8^+$ T cells do not express IFN-γ at levels as high as $CD4^+$ T cells, they are shown here and elsewhere to be capable of *Brucella*-specific killing (22).

We began our studies with the goal of identifying *B. melitensis*-specific $CD8^+$ T-cell epitopes and the immunogenic proteins that contain them. The epitopes described here represent the first characterized $CD8^+$ T-cell epitopes identified in H-2^d mice, a common and cost-effective model for studying *B. melitensis* infection and immunity. Importantly, these epitopes were able to induce $CD8^+$ T cells that kill in vivo. Our data show that we have validated a promising strategy of identifying immunogenic determinants from the large *Brucella* genome. This strategy, in concert with our microarray data, is an approach that opens the door to looking at potential vaccine constructs based not only on the immunogenicity of the epitopes but also their expression kinetics.

Proteomic analysis of *B. suis* published recently by others has shown by two-dimensional DIGE that the homologue to BMEI 1961 in *B. suis*, BR2169, significantly increases protein expression 2.25-fold after the infection of macrophages (2). This parallels the increase seen in our microarray data of BMEI 1961 postinfection of macrophages. Although BMEII 0819 decreased 22 h postinfection, this does not reduce its importance in the course of infection and the host response. This protein may play a role in the early immune response or in the extracellular environment, and this phase of pathogenesis should not be ignored since antigenic epitopes from this phase may be important for targeting cells presenting the first wave of bacteria to be processed upon infection. Because we do not yet know the basal levels of expression of these proteins, high levels of protein may be available for processing by the antigen presentation machinery of the host. Microarrays done under different experimental conditions, i.e., infection times and cell types, performed in concert with qRT-PCR will fill in the gaps of what we know about the expression of our epitope-containing proteins. Future work will investigate the kinetic expression of epitope-containing proteins over the course of infection,

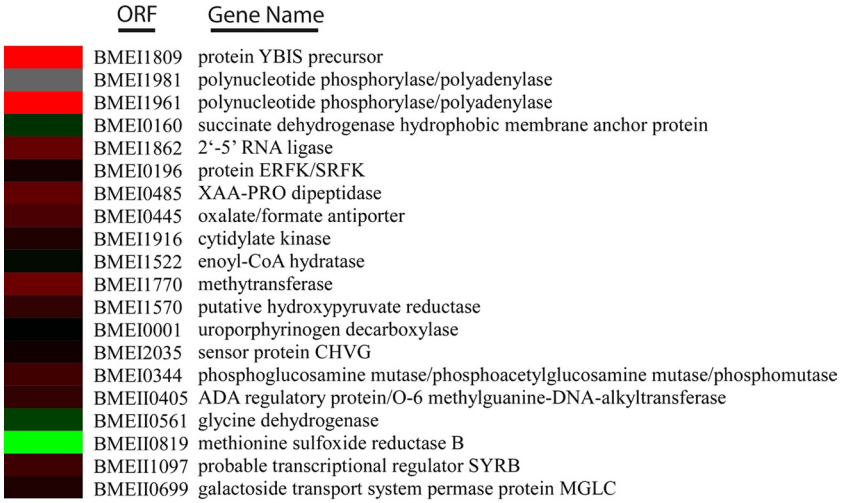

FIG. 5. Microarray. A heat map of *B. melitensis* genes that we predicted to contain epitopes altered during 24 h infection of mouse macrophages compared to cultures grown in broth was prepared. Each horizontal line represents one gene. Log_2 ratios are color coded, as shown in the bar. Green indicates less abundance of the target RNA compared to control RNA (1.e. downregulated), whereas red represents greater abundance of the target RNA compared to control RNA (i.e., upregulated genes).

contributing to the development of a multivalent vaccine that includes epitopes from *B. melitensis* genes expressed at different stages of infection. This construct would have the advantage of inducing $CD8^+$ T cells with different specificities that cover the continuum of protein expression during the infection of host cells.

Considering that long-term protection to *Brucella* is either not complete or largely not addressed in studies of single whole-protein and polymeric vaccines, we do not anticipate that complete protection might be engendered by the single epitopes presented here (1, 17, 31). Rather, these data allow the development of tetramers to track the *Brucella*-specific $CD8^+$ T-cell response, as well as lay the groundwork for potential multivalent peptide vaccines of the future. Protection studies have not yet been done, which will also include choosing the correct vector and/or adjuvant system, since these can impact the responding T-cell clones (23). Although our analysis predicted more than 6,000 MHC class I *B. melitensis* epitopes, it is likely that this large bacterial genome encodes many more. One of the epitopes characterized here, NGSSS MATV, was not predicted to bind either class I allele and was originally chosen as a representative nonbinder, revealing that there remains some weakness in prediction methods. The specificity and relative strength of these epitopes became apparent in the in vivo functional killing assays, since the two epitopes were able to induce specific killing.

Future studies will include challenge with virulent *Brucella* spp., dissection of the memory response, and work with mice transgenic for human MHC alleles to investigate the possibility of immunogenicity in human infection. We will also continue to identify other $CD8^+$ T-cell epitopes, as well as antibody epitopes. The epitopes that have been identified in these studies are also predicted to bind various human class I MHC alleles (45). RYCINSASL is predicted to bind HLA A*2403 and HLA A*3201. NGSSSMATV is predicted to bind HLA A*0202, HLA A*0203, HLA A*6802, and HLA A*0206. Interestingly, these epitopes are probably cross-reactive because these proteins are conserved with intact epitopes in *B. abortus*, *B. canis*, *B. suis*, and *B. ovis*.

The field of *Brucella* vaccine research has recently seen exciting advancements, with the introduction of several novel investigations into mechanisms of *Brucella* antigen delivery and the induction of cellular immunity (6, 21). In addition, our group and others are looking further into *Brucella* attenuation as an effective vaccine development strategy (5, 29). Identifying and dissecting *B. melitensis* $CD8^+$ T-cell epitopes that trigger host immunity in vivo are critical to assembling future *Brucella* vaccines, and we will be able to investigate the effectiveness of a multivalent peptide vaccine that includes multiple *Brucella* epitopes. To produce a safe, viable human vaccine, mechanisms of host immunity need to be clarified. The findings presented here contribute indispensable immunogenic epitopes as the newest tools for tracking the expansion, contraction, and memory development of the *Brucella*-specific response.

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