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Searching for Borrelial T cell Epitopes Associated with Antibiotic-Refractory Lyme Arthritis

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Abstract

Antibiotic-refractory Lyme arthritis is believed to result from an infection-induced autoimmune response triggered by the spirochete *Borrelia burgdorferi* (*Bb*). Disease susceptibility is associated with the HLA alleles DRB1*0101, 0401, 0402, 0404, 0405 and DRB5*0101, and all these MHC molecules bind the *Bb* epitope OspA₁₆₃₋₁₇₅. However, not all patients have a proliferative response to this epitope. To identify other possible *Bb* epitopes involved in this disease process, the algorithm TEPITOPE was used to scan 17 immunogenetic *Bb* proteins for potential T cell epitopes with a refractory arthritis-associated MHC binding profile, and the *Bb* proteome was searched for peptides with sequence homology to OspA₁₆₃₋₁₇₅. Sixteen promising T epitopes were identified and their MHC binding profiles to 13 MHC molecules were verified using *in vitro* MHC/peptide binding assays. One peptide, BBK32₃₉₂₋₄₀₄, had a strong refractory-arthritis associated MHC binding profile, and another GK₂₉₇₋₃₀₆ shared sequence homology to OspA₁₆₃₋₁₇₅. However, patient cells did not proliferate in response to either peptide making it highly unlikely they were involved in a refractory course. A comparison of the *in silico* and *in vitro* results revealed that TEPITOPE correctly predicted 74% of the *in vitro* binding peptides, but it incorrectly predicted that 44% of the *in vitro* nonbinding peptides would bind. For a particular MHC molecule, concordance between the *in silico* and *in vitro* results varied anywhere between 33% and 100%. Therefore, while additional *Bb* epitopes may be involved in the development of antibiotic-refractory Lyme arthritis, recognition of OspA₁₆₃₋₁₇₅ remains the only known *Bb* epitope associated with this disease.

1. Introduction

Lyme arthritis, which is caused by the tick-borne spirochete, *Borrelia burgdorferi* (*Bb*), is characterized by intermittent or persistent arthritis in a few large joints, particularly the knee. (Steere, 2001; Steere et al., 1987) In most patients, the arthritis can be treated successfully with either a 1 or 2-month course of oral doxycycline or a 2 or 4-week course of intravenous (IV) ceftriaxone. (Dattwyler et al., 1988; Steere et al., 1994) In rare cases however, synovitis persists for months or even several years despite treatment with ≥ 2 months of oral antibiotics, ≥ 1 months of IV antibiotics, or usually both. (Steere and Angelis, 2006; Steere et al., 1994) Since PCR results for *Bb* DNA in joint fluid are almost always negative in the post-antibiotic period

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(Carlson et al., 1999; Nocton et al., 1994; Steere and Angelis, 2006) and since cellular and humoral immune responses to *Bb* antigens begin to decline soon after antibiotic treatment (Kannian et al., 2007a; Kannian et al., 2007b), antibiotic therapy appears to result in the nearly complete or total eradication of spirochetes from the joint, yet synovial inflammation still persists. This disease course, which has been termed antibiotic-refractory Lyme arthritis, is thought to be the result of an infection-induced autoimmune response. (Steere and Glickstein, 2004)

A common feature of many autoimmune diseases is a link with specific HLA genotypes. Antibiotic-refractory Lyme arthritis is associated with the six HLA-DR molecules (DRB1*0401, 0101, 0404, 0405, 0402 and DRB5*0101) that bind the *Bb* epitope outer-surface protein A₁₆₅₋₁₇₃ (OspA₁₆₅₋₁₇₃) (Steere et al., 2006) and with T cell reactivity with this epitope. (Chen et al., 1999) In contrast, four MHC molecules (DRB1*0301, 0801, 1101 and 1104) that do not bind this OspA epitope are associated with an antibiotic-responsive course. How T cell reactivity to OspA₁₆₅₋₁₇₃ might be involved in antibiotic-refractory Lyme arthritis is presently unknown. Molecular mimicry between the OspA epitope and a sequence in human LFA-1 was proposed. (Gross et al., 1998) However, in subsequent studies, the LFA-1 peptide, as well as other human peptides with OspA₁₆₅₋₁₇₃ sequence homology, acted as only weak agonists in a small number of patients with refractory arthritis (Drouin et al., 2008; Trollmo et al., 2001), suggesting that they are unlikely to be relevant autoantigens in this illness. Furthermore, in a recent analysis using T cell tetramer reagents, three of seven DRB1*0401-positive patients with refractory arthritis lacked detectable OspA₁₆₅₋₁₇₃-specific T cells or proliferative responses to this epitope, whereas three of six patients with antibiotic-responsive arthritis had these OspA responses. (Kannian et al., 2007b) Thus, reactivity with this epitope, by itself, cannot explain all cases of antibiotic-refractory Lyme arthritis.

An alternative hypothesis is that T cell recognition of OspA₁₆₅₋₁₇₃, as well as certain other *Bb* epitopes, induce high levels of pro-inflammatory cytokines, which lead to bystander activation of autoreactive T cells and persistent synovitis. (Benoist and Mathis, 2001) Consistent with this hypothesis, we recently reported that patients with antibiotic-refractory arthritis had significantly higher levels of T_H1 chemoattractants and cytokines in joint fluid, particularly CXCL9 and IFN- γ , than patients with antibiotic-responsive arthritis. (Shin et al., 2007) Moreover, in patients with refractory arthritis, the levels of these chemokines and cytokines in synovial fluid remained high or even increased in the post-antibiotic period, while cellular and humoral reactivity with borrelial proteins, including OspA, declined. However, thus far, no borrelial epitope, other than OspA₁₆₅₋₁₇₃, has been identified that has an antibiotic-refractory arthritis-associated DRB binding profile and induces a T cell response in patients with refractory arthritis.

Bioinformatics programs can be used to predict T cell epitopes and their binding potential to DRB molecules. For example, the algorithm TEPITOPE, which is based on *in vitro* MHC/peptide binding of designer peptides, can predict the binding potential of T cell epitopes to 25 different DRB molecules. (Hammer et al., 1994; Hammer et al., 1997; Sturniolo et al., 1999) However, the ability of this program to identify potential disease-associated epitopes with a specific MHC binding profile has not yet been tested.

In the current study, we used TEPITOPE to screen 17 known immunogenic *B. burgdorferi* proteins for T cell epitopes that were predicted to bind preferentially to antibiotic-refractory arthritis-associated DRB molecules. In addition, we searched the *B. burgdorferi* proteome for proteins with sequence homology to OspA₁₆₅₋₁₇₃. Promising epitopes identified *in silico* were tested for actual binding *in vitro* to 13 available HLA-DR molecules, including the six associated with antibiotic-refractory arthritis. One of the epitopes identified by the algorithm, BBK32₃₉₂₋₄₀₄ (*Bb* fibronectin binding protein), was demonstrated to have an antibiotic-

refractory arthritis-associated DRB binding profile *in vitro*, while GK₂₉₇₋₃₀₆ (*Bb* glycerol kinase) had a high level of sequence homology with OspA₁₆₅₋₁₇₃. However, whereas many patients' cells recognized the OspA epitope, patients' T cells did not have a proliferative response to either the BBK32 or GK peptides.

2. Materials and methods

2.1 Computer databases and binding algorithms

Bb protein sequences were identified using PubMed and accession numbers are given in Table 1. Using TEPITOPE 2000 as a primary screening tool (on line at www.vaccinome.com), putative T cell epitopes were identified in 17 known immunogenic *Bb* proteins, and their predicted binding by 13 HLA-DRB molecules was assessed. In addition, the complete *Bb* B31 proteome was searched for peptides that had at least four amino acid residues in common with OspA₁₆₅₋₁₇₃ (YVLEGTLLTA) using blastp and the filter disabled

(
http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?taxid=%20Environmental&taxidinf=enviroinfo&selectall
).

2.2 HLA-DR molecules and MHC/peptide binding assays

For *B. burgdorferi* T cell epitopes with promising predicted MHC binding profiles, actual peptide binding was determined *in vitro* using the same 13 HLA-DRB molecules that were assessed *in silico*. Based on a previous analysis of DRB binding of OspA₁₆₃₋₁₇₅ to these 13 DRB molecules (Steere et al., 2006), an antibiotic-refractory arthritis-associated DRB binding profile was defined as one in which a given peptide was bound by the HLA-DRB1*0101, 0401, 0402, 0404, or 0405 molecule or by the DRB5*0101 or DRB1*1501 molecule, but not by the DRB1*0301, 1101 and 1104 molecules. Since antibiotic-refractory arthritis was not clearly associated with either the DRB1*0102 or the DRB1*0701/DRB4*0101 molecules, these molecules were not included in the definition.

MHC/peptide binding assays were performed by incubating recombinant DRB molecules overnight with biotinylated peptides as previously described (Steere et al., 2006). The following day, the MHC/peptide complexes were captured on 96-well plates coated with the monoclonal antibody L243, a HLA-DR specific antibody (American Type Culture Collection, Rockville, MD). After washing to remove unbound peptides, europium-labeled streptavidin (Wallac/PerkinElmer) was added. The plate was washed a final time and fluorescence was measured with a Victor² 1420 multilabel counter (Wallac/PerkinElmer).

All positive control peptides gave markedly positive results. The sequences of the positive control peptides were 0102-PC, PKYVKLNALKLAT for the DRB1*01 molecules; influenza NSI₃₂₋₄₅, FLDRLRRDQRSLRG for 0301; GAD65₅₅₇₁, NFIRMVMSNPAAT for the 04 molecules, 1501 and B4*0101; 0701-PC, TSLYVRTSSFVIVSI for 0701; 1104-PC, PKYVKLNKLKLSAT for 1101 and 1104; and HA₃₀₇₋₃₁₉, PKYVKQNTLKLAT for B5*0101. OspA_{165A}, KGAVLEGTLLTAEK was used as a negative control peptide with all MHC molecules except the 04 molecules in which 04-NC, VSAATRGSLLQATV was used (data not shown).

2.3 Proliferation Assays

To determine whether patients with Lyme arthritis had T cell reactivity with implicated *Bb* peptides, cells were tested from 15 patients, 8 with antibiotic-refractory arthritis and 7 with antibiotic-responsive arthritis, who were seen during a 7-month period between March and September 2005. All patients participated in a study, "Immunity in Lyme Arthritis", which was approved by the Human Investigations Committees at Massachusetts General Hospital. T

cell proliferation assays using bulk lymphocytes were performed as previously described (Chen et al., 1999). Briefly, PBMC (2×10^5 cells per well) were incubated with peptides (1 μ M final concentration) in duplicate wells. After five days, 0.5 μ Ci of 3 H-thymidine (PerkinElmer) was added and cells were harvested 16-18 hours later. Incorporation of 3 H-thymidine was measured using a TopCount scintillation counter (PerkinElmer).

2.4 Comparing *in silico* with *in vitro* results

The MHC/peptide binding results predicted by TEPITOPE were compared with those obtained by *in vitro* MHC/peptide binding assays. First, the result of each *in vitro* assay was categorized as to whether the target peptide bound or was not bound by the DR molecule. Binding was defined as a fluorescent value greater than 3SD above the mean value of the negative control peptides ($\geq 90,000$ relative fluorescent units). The observed results were compared with the predictions obtained using three threshold settings: a) $\geq 3\%$ - high stringency; $\geq 10\%$ - low stringency or c) <10 - not predicted to bind. In addition, the *in silico* and *in vitro* results were compared for each MHC molecule using the TEPITOPE threshold settings: a) 3% - high stringency; b) 10% - low stringency; and c) $>10\%$ - not predicted to bind. The level of concordance between the two methods was then computed.

3. Results

3.1 Bb T cell epitopes predicted to have an antibiotic-refractory Lyme arthritis binding profile

To identify *Bb* epitopes, in addition to OspA₁₆₅₋₁₇₃, with an antibiotic-refractory associated DRB binding profile, 17 known immunogenic *Bb* proteins (Akin et al., 2001; Bacon et al., 2003; Dressler et al., 1993; Heikkila et al., 2003; Salazar et al., 2005) (Table 1) were screened with the bioinformatics program TEPITOPE. This program scans proteins for peptides predicted to bind one or more of 25 different HLA-DR molecules, and it ranks peptide binding according to stringency (threshold parameter), which can be set anywhere from 1 (the most stringent) to 10 (the least stringent). In our initial screen, we used a threshold of 3, as recommended the program's designers, to keep the number of false-positive results reasonably low. (Bian et al., 2003)

In this initial screen of the 17 known immunogenic *Bb* proteins, 43 epitopes were predicted to be presented by the HLA-DR*0401 and 0101 molecules, the two MHC molecules most strongly associated with antibiotic-refractory Lyme arthritis. (Steere et al., 2006) All but two of the proteins, OspB and VlsE, contained such peptides. When these 43 epitopes were further assessed to determine their predicted binding profile to 10 additional HLA-DR molecules, 35 peptides, including OspA₁₆₃₋₁₇₅, demonstrated a high probability of presentation by four or more refractory arthritis-associated DRB molecules.

In addition to the screening of known immunogenic *Bb* proteins, we searched the borrelial proteome for peptides with sequence homology with the OspA₁₆₅₋₁₇₃ epitope, which is currently the only borrelial peptide known to have a refractory arthritis-associated HLA-DR binding profile. From this search, peptides from four different proteins were identified with homology to four or more of the OspA₁₆₅₋₁₇₃ residues: glycerol kinase (GK₂₉₇₋₃₀₆) and three hypothetical proteins (Table 1). GK₂₉₇₋₃₀₆, a housekeeping enzyme also expressed in human cells, shared identity with OspA₁₆₅₋₁₇₃ at five of the nine core residues, including two T cell contact sites. In addition, three of the six refractory arthritis-associated DRB molecules were predicted to bind the GK₂₉₇₋₃₀₆ epitope. In contrast, two of the hypothetical proteins were predicted not to bind any of the refractory arthritis-associated MHC molecules due to poor P1 anchor residues, and the third had a lysine at the P9 position, which was likely to inhibit binding to the shallow, hydrophobic P9 pockets of the DRB1*0101 and 0401 molecules. (Hill et al., 1994)

3.2 In vitro MHC/peptide binding assays of Bb epitopes

Because the availability of sufficient quantities of recombinant MHC molecules was limiting, 15 of the 35 epitopes from known immunogenic proteins predicted to bind at least four of the six refractory arthritis-associated DRB molecules (Table 2), along with the GK₂₉₇₋₃₀₆ epitope (Table 3), were selected for *in vitro* MHC/peptide binding studies to 13 available HLA-DRB molecules. These 13 MHC molecules represent nearly 80% of the DRB molecules found in patients with Lyme arthritis. (Steere et al., 2006) Twelve of these 13 DRB molecules were also part of the TEPITOPE algorithm; the program does not predict binding to the DRB4*0101 molecule.

The results of the binding assays with the OspA₁₆₃₋₁₇₅ peptide were the same as in our previous analysis. (Steere et al., 2006) Six DRB molecules, which are found more commonly in patients with antibiotic-refractory arthritis (DRB1*0401, 0101, 0404, 0405, 0402 and DRB5*0101), bound OspA₁₆₃₋₁₇₅, whereas three molecules, which are found more often in patients with antibiotic-responsive arthritis (DRB1*0301, 1101, and 1104), did not (Figure 1). Regarding the MHC molecules found nearly equally in both patient groups, the DRB1*0102 molecule bound OspA₁₆₃₋₁₇₅ weakly, whereas both members of the disequilibrium pair B1*0701/B4*0101 did not.

Of the 15 newly tested borrelial epitopes, only one, fibronectin binding protein₃₉₂₋₄₀₄ (BBK32₃₉₂₋₄₀₄), had a strong refractory arthritis-associated DRB binding profile (Figure 1). As with the OspA peptide, the BBK32 peptide bound the DRB1*0101 molecule, the four 04 molecules and the B5*0101 molecule. Moreover, in contrast with the OspA peptide, limited binding was detected to both members of the DRB disequilibrium pair DRB1*0701/B4*0101, which is found slightly more often in patients with refractory arthritis (Steere et al., 2006). The only deviation from the antibiotic-refractory DRB profile was the binding of BBK32₃₉₂₋₄₀₄ to the DRB1*1101 molecule, which is found more often in antibiotic-responsive patients. Though OspA₁₆₃₋₁₇₅ and BBK32₃₉₂₋₄₀₄ peptides had very similar DRB binding profiles, their nine core amino acid sequences were quite dissimilar with seven non-conservative substitutions including four at MHC contact sites.

Several other epitopes came close to having an antibiotic-refractory arthritis-associated DRB binding profile, but lacked strong binding to one or more of the critical DRB molecules. For example, OSpC₁₂₋₂₄ bound five of the six refractory arthritis-associated molecules, rather like the OspA peptide, but it did not bind well to the DRB1*0101 molecule. GK₂₉₇₋₃₀₆ showed strong to weak binding to four of the six refractory arthritis-associated MHC molecules, but it lacked binding to the DRB1*0402 and 0404 molecules. The remaining 10 borrelial peptides also lacked binding to at least one of the refractory arthritis-associated DRB molecules. OSpE₁₂₋₂₄ and OSpF₃₋₁₅ had low-level binding to all of the MHC molecules tested, suggesting that this binding pattern was non-specific. Thus, only OspA₁₆₅₋₁₇₃ and BBK32₃₉₂₋₄₀₄ bound well to all six antibiotic-refractory arthritis-associated DRB molecules.

3.3 Recognition of Bb epitopes by patients' T cells

Because availability of patients' T cells is limited and because these cells cannot be replenished, we determined the immunogenicity of only OspA₁₆₀₋₁₇₇, BBK32₃₉₂₋₄₀₄ and GK₂₉₅₋₃₀₇. First, fresh PBMC from patients with Lyme arthritis seen over a five month period, five with antibiotic-refractory arthritis and six with antibiotic-responsive arthritis were stimulated with OspA₁₆₀₋₁₇₇ and BBK32₃₉₂₋₄₀₄ in standard proliferation assays (Figure 2A). T cells from four of the five patients with refractory arthritis and three of the six patients with responsive arthritis had proliferative responses to OspA₁₆₀₋₁₇₇ that were at least two times above background. However, cells from none of the 11 patients proliferated in response to the BBK32 peptide.

Furthermore, PBL from 10 normal control subjects did not proliferate in response to either antigen.

Because the GK₂₉₅₋₃₀₇ peptide was identified after the BBK32₃₉₂₋₄₀₄ peptide, frozen cells from the same 11 patients that had been tested for responses to the BBK32 peptide and frozen cells from eight normal control subjects were tested for reactivity with the GK peptide. While two of the patients had a proliferative response to the OspA peptide, none of the patients or control subjects recognized GK₂₉₅₋₃₀₇. Thus, neither the BBK32 peptide nor the GK peptide seemed likely to be involved in an antibiotic-refractory disease course.

3.4 Comparison of computer binding predictions with *in vitro* MHC/peptide binding results

To assess the predictive powers of TEPITOPE, we compared the *in silico* MHC/peptide binding results (Tables 2 & 3) with the *in vitro* results (Figure 1). (DRB4*0101 was not included in this analysis as it was not part of the TEPITOPE program). To perform this analysis, *in vitro* MHC/peptide binding had to be classified as positive or negative. Therefore, a positive response was defined as a fluorescent value greater than three standard deviations above the mean value obtained with the negative control peptides ($\geq 90,000$ relative fluorescent units). According to this criterion, 117 of the 192 *in vitro* MHC/peptide binding assays performed had positive results. When these results were compared with TEPITOPE's predictions at a threshold of 3 (which was used for the initial screen), the program correctly predicted 74% (87/117) of the binding peptides (Figure 3). At the least stringent threshold setting of 10, TEPITOPE correctly identified 97% (113/117) of the binding peptides. However, among the 75 *in vitro* assays in which peptide binding did not occur, TEPITOPE incorrectly predicted that 44% (33/75) of these peptides would bind at a threshold of 3 and 87% (65/75) at a threshold of 10.

The predictive power of the algorithm was also analyzed individually for the 12 MHC molecules tested (Table 4). At a threshold setting of 3, concordance between the *in vitro* and *in silico* results ranged from 33% to 100%. TEPITOPE's predictions were highest (90 to 100% concordance) for the DRB1*0402, 0101, 0404 and B5*0101 molecules; intermediate (60 to 90% concordance) for the DRB1*0102, 0401, 1501, and 0405 molecules; and lowest ($\leq 50\%$ concordance) for the DRB1*1101, 1104, 0701 and 0301 molecules. Interestingly, although the DRB5*0101 molecule bound all six peptides that it was predicted to bind, it also bound two peptides it was not predicted to bind at any threshold setting. Thus, the predictive power of the virtual matrix varied among MHC molecules.

4. Discussion

We previously showed a highly significant association between antibiotic-refractory Lyme arthritis and specific HLA-DR molecules that bind the borrelial epitope OspA₁₆₃₋₁₇₅. (Steere et al., 2006) However, since not all patients with antibiotic-refractory Lyme arthritis have reactivity with this peptide, we searched here for other stimulatory borrelial epitopes with an antibiotic-refractory arthritis-associated DRB binding profile.

The number of potential epitopes is enormous. The Institute of Genomic Research (TIGR) calculates that the *Bb* proteome contains 1,740 known or hypothetical proteins, and each protein may have multiple T cell epitopes. Thus, we restricted our search to epitopes in 17 *Bb* proteins, which included most of the spirochete's known immunogenic proteins and those epitopes with OspA₁₆₃₋₁₇₅ sequence homology. Still, even this more restricted search included more epitopes than would be practical to test using *in vitro* MHC/peptide binding and T cell proliferation assays. Therefore, to further focus the search, we used the computer algorithm TEPITOPE to predict the MHC-peptide binding patterns of these borrelial epitopes. Of the 35 epitopes identified by the algorithm to bind at least four of the six antibiotic-refractory arthritis-

associated DRB molecules, including the highly associated DRB1*0101 and 0401 molecules, 16 were selected for further analysis.

Next, we determined the *in vitro* the MHC/peptide binding profiles of these 16 borrelial epitopes to 13 different HLA-DRB molecules, which required a total of 208 individual binding assays. Of the 16 borrelial peptides tested, only one (BBK32₃₉₂₋₄₀₄) had a strong antibiotic-refractory arthritis-associated DRB binding profile. In fact, its binding profile correlated with the HLA data even better than that of OspA₁₆₅₋₁₇₃ (Steere et al., 2006), since the genetically linked DRB1*0701 and DRB4*0101 molecules, which are found slightly more frequently in patients with antibiotic-refractory arthritis, bound BBK32₂₉₂₋₄₀₄, but not OspA₁₆₅₋₁₇₃. Patients with early Lyme disease frequently have an antibody response to the BBK32 protein and higher antibody responses correlate with less severe disease. (Akin et al., 1999) However, at this later stage of the illness, it appears that Lyme arthritis patients lack reactivity with this particular BBK32 peptide.

In addition, we looked for borrelial peptides with partial sequence homology with OspA₁₆₅₋₁₇₃. One possible epitope (glycerol kinase₂₉₇₋₃₀₆) was identified. As a key enzyme that shuttles glucose into the glycolytic pathway, glycerol kinase is expressed in both bacterial and human cells making it an interesting candidate molecular mimic. However, *in vitro* MHC/peptide binding assays demonstrated that the Bb GK₂₉₇₋₃₀₆ peptide was bound by only four of the six MHC molecules associated with refractory Lyme arthritis, and it did not stimulate patients' T cells. Therefore, we concluded that neither the BBK32 epitope nor the GK epitope was likely to be important in the pathogenesis of antibiotic-refractory Lyme arthritis.

It is certainly possible that borrelial epitopes other than OspA₁₆₃₋₁₇₅ might have a pathogenic role in antibiotic-refractory Lyme arthritis, but they may not bind all of the DRB molecules *in vitro* that are associated with a refractory course. First, the *in vitro* binding assay may not be sensitive enough to detect low-level binding that is still sufficient to activate patients' T cells. For example, we previously found that a human homologue of OspA₁₆₅₋₁₇₃, called MAWDBP₂₇₆₋₂₈₈, did not bind *in vitro* to the DRB1*1501 molecule or the genetically linked DRB5*0101 molecule, but this peptide stimulated T cells in some patients with Lyme arthritis who expressed these molecules (Drouin et al., 2008). Second, if certain borrelial peptides trigger particularly high pro-inflammatory responses, or even serve as a molecular mimics, they might do so in association with some, but not all, of the refractory arthritis-associated DRB molecules. Ultimately, the critical test always will be whether a given peptide stimulates patients' T cells. However, because of the limited availability of patients' cells and the lack of an animal model of antibiotic-refractory Lyme arthritis, it is currently possible to do such testing with only a limited number of borrelial peptides.

In this study, we were able to compare the *in silico* results to the *in vitro* results over a wide range of MHC molecules. Interestingly, we found that the predictive powers of TEPITOPE varied significantly depending on the DRB molecule. For example, at a threshold setting of 3, *in silico* and *in vitro* results were 94% concordant with the DRB1*0101 molecule, yet only 69% concordant with the 0401 molecule. Consequently, our initial screen, which was predicated on TEPITOPE identifying peptides bound by both DRB1*0101 and 0401, may have overlooked some candidate T cell epitopes. In other studies, TEPITOPE has been used successfully to identify biologically active promiscuous T cell epitopes from a single protein or small family of proteins in the context of cancer (Manici et al., 1999; Sturniolo et al., 1999), infectious disease (Panigada et al., 2002), or autoimmunity (Hammer et al., 1997). However, the algorithm seems less effective at identifying T cell epitopes with a specific MHC binding profile from a large array of proteins. Therefore, we concluded that *in vitro* MHC/peptide binding assays must still be performed to confirm peptide binding.

In summary, using a combination of the TEPITOPE program, *in vitro* MHC/peptide binding assays, and sequence alignment algorithms, we identified one new borrelial epitope (BBK32₃₉₂₋₄₀₄), in addition to OspA₁₆₃₋₁₇₅, that had a strong antibiotic-refractory arthritis-associated DRB binding profile, and another (GK₂₉₇₋₃₀₆) with sequence homology with the OspA epitope. However, T cells from patients with Lyme arthritis did not proliferate in response to either of these epitopes. Even so, other as yet unidentified borrelial epitopes may play a pathogenic role in the development of antibiotic-refractory Lyme arthritis.

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References

- Akin E, Aversa J, Steere AC. Expression of adhesion molecules in synovia of patients with treatment-resistant Lyme arthritis. *Infect Immun* 2001;69:1774–80. [PubMed: 11179355]
- Akin E, McHugh GL, Flavell RA, Fikrig E, Steere AC. The immunoglobulin (IgG) antibody response to OspA and OspB correlates with severe and prolonged Lyme arthritis and the IgG response to P35 correlates with mild and brief arthritis. *Infect Immun* 1999;67:173–81. [PubMed: 9864212]
- Bacon RM, Biggerstaff BJ, Schrieffer ME, Gilmore RD Jr, Philipp MT, Steere AC, Wormser GP, Marques AR, Johnson BJ. Serodiagnosis of Lyme disease by kinetic enzyme-linked immunosorbent assay using recombinant VlsE1 or peptide antigens of *Borrelia burgdorferi* compared with 2-tiered testing using whole-cell lysates. *J Infect Dis* 2003;187:1187–99. [PubMed: 12695997]
- Benoist C, Mathis D. Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry? *Nat Immunol* 2001;2:797–801. [PubMed: 11526389]
- Bian H, Reidhaar-Olson JF, Hammer J. The use of bioinformatics for identifying class II-restricted T-cell epitopes. *Methods* 2003;29:299–309. [PubMed: 12725795]
- Carlson D, Hernandez J, Bloom BJ, Coburn J, Aversa JM, Steere AC. Lack of *Borrelia burgdorferi* DNA in synovial samples from patients with antibiotic treatment-resistant Lyme arthritis. *Arthritis Rheum* 1999;42:2705–9. [PubMed: 10616021]
- Chen J, Field JA, Glickstein L, Molloy PJ, Huber BT, Steere AC. Association of antibiotic treatment-resistant Lyme arthritis with T cell responses to dominant epitopes of outer surface protein A of *Borrelia burgdorferi*. *Arthritis Rheum* 1999;42:1813–22. [PubMed: 10513794]
- Dattwyler RJ, Halperin JJ, Volkman DJ, Luft BJ. Treatment of late Lyme borreliosis--randomised comparison of ceftriaxone and penicillin. *Lancet* 1988;1:1191–4. [PubMed: 2897008]
- Dressler F, Whalen JA, Reinhardt BN, Steere AC. Western blotting in the serodiagnosis of Lyme disease. *J Infect Dis* 1993;167:392–400. [PubMed: 8380611]
- Drouin EE, Glickstein L, Kwok WW, Nepom GT, Steere AC. Human homologues of a *Borrelia* T cell epitope associated with antibiotic-refractory Lyme arthritis. *Mol Immunol* 2008;45:180–9. [PubMed: 17555819]
- Gross DM, Forsthuber T, Tary-Lehmann M, Etling C, Ito K, Nagy ZA, Field JA, Steere AC, Huber BT. Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis. *Science* 1998;281:703–6. [PubMed: 9685265]
- Hammer J, Bono E, Gallazzi F, Belunis C, Nagy Z, Sinigaglia F. Precise prediction of major histocompatibility complex class II-peptide interaction based on peptide side chain scanning. *J Exp Med* 1994;180:2353–8. [PubMed: 7964508]
- Hammer J, Sturniolo T, Sinigaglia F. HLA class II peptide binding specificity and autoimmunity. *Adv Immunol* 1997;66:67–100. [PubMed: 9328640]
- Heikkila T, Huppertz HI, Seppala I, Sillanpaa H, Saxen H, Lahdenne P. Recombinant or peptide antigens in the serology of Lyme arthritis in children. *J Infect Dis* 2003;187:1888–94. [PubMed: 12792865]

- Hill CM, Liu A, Marshall KW, Mayer J, Jorgensen B, Yuan B, Cubbon RM, Nichols EA, Wicker LS, Rothbard JB. Exploration of requirements for peptide binding to HLA DRB1*0101 and DRB1*0401. *J Immunol* 1994;152:2890–8. [PubMed: 8144889]
- Kannian P, Drouin EE, Glickstein L, Kwok WW, Nepom GT, Steere AC. Decline in the Frequencies of *Borrelia burgdorferi* OspA₁₆₁ 175-Specific T Cells after Antibiotic Therapy in HLA-DRB1*0401-Positive Patients with Antibiotic-Responsive or Antibiotic-Refractory Lyme Arthritis. *J Immunol* 2007a;179:6336–42. [PubMed: 17947711]
- Kannian P, McHugh G, Johnson BJB, Bacon RM, Glickstein LJ, Steere AC. Antibody responses to *Borrelia burgdorferi* in patients with antibiotic-refractory, antibiotic-responsive, or non-antibiotic-treated Lyme arthritis. *Arthritis Rheum*. 2007bIn press
- Manici S, Sturniolo T, Imro MA, Hammer J, Sinigaglia F, Noppen C, Spagnoli G, Mazzi B, Bellone M, Dellabona P, Protti MP. Melanoma cells present a MAGE-3 epitope to CD4(+) cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. *J Exp Med* 1999;189:871–6. [PubMed: 10049951]
- Nocton JJ, Dressler F, Rutledge BJ, Rys PN, Persing DH, Steere AC. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. *N Engl J Med* 1994;330:229–34. [PubMed: 8272083]
- Panigada M, Sturniolo T, Besozzi G, Boccieri MG, Sinigaglia F, Grassi GG, Grassi F. Identification of a promiscuous T-cell epitope in *Mycobacterium tuberculosis* Mce proteins. *Infect Immun* 2002;70:79–85. [PubMed: 11748166]
- Salazar CA, Rothemich M, Drouin EE, Glickstein L, Steere AC. Human Lyme arthritis and the immunoglobulin G antibody response to the 37-kilodalton arthritis-related protein of *Borrelia burgdorferi*. *Infect Immun* 2005;73:2951–7. [PubMed: 15845501]
- Shin JJ, Glickstein L, Steere AC. High levels of inflammatory chemokines and cytokines in joint fluid and synovial tissue throughout the course of antibiotic-refractory Lyme arthritis. *Arthritis Rheum* 2007;56:1325–1335. [PubMed: 17393419]
- Steere AC. Lyme disease. *N Engl J Med* 2001;345:115–25. [PubMed: 11450660]
- Steere AC, Angelis SM. Therapy for Lyme arthritis: strategies for the treatment of antibiotic-refractory arthritis. *Arthritis Rheum* 2006;54:3079–86. [PubMed: 17009226]
- Steere AC, Glickstein L. Elucidation of Lyme arthritis. *Nat Rev Immunol* 2004;4:143–52. [PubMed: 15040587]
- Steere AC, Klitz W, Drouin EE, Falk BA, Kwok WW, Nepom GT, Baxter-Lowe LA. Antibiotic-refractory Lyme arthritis is associated with HLA-DR molecules that bind a *Borrelia burgdorferi* peptide. *J Exp Med* 2006;203:961–71. [PubMed: 16585267]
- Steere AC, Levin RE, Molloy PJ, Kalish RA, Abraham JH 3rd, Liu NY, Schmid CH. Treatment of Lyme arthritis. *Arthritis Rheum* 1994;37:878–88. [PubMed: 8003060]
- Steere AC, Schoen RT, Taylor E. The clinical evolution of Lyme arthritis. *Ann Intern Med* 1987;107:725–31. [PubMed: 3662285]
- Sturniolo T, Bono E, Ding J, Raddrizzani L, Tuereci O, Sahin U, Braxenthaler M, Gallazzi F, Protti MP, Sinigaglia F, Hammer J. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat Biotechnol* 1999;17:555–61. [PubMed: 10385319]
- Trollmo C, Meyer AL, Steere AC, Hafler DA, Huber BT. Molecular mimicry in Lyme arthritis demonstrated at the single cell level: LFA-1 alpha L is a partial agonist for outer surface protein A-reactive T cells. *J Immunol* 2001;166:5286–91. [PubMed: 11290815]

Abbreviations

OspA

outer-surface protein A

Bb

Borrelia burgdorferi

BBK32

GK

fibronectin binding protein

glycerol kinase

Figure 1. *In vitro* binding profiles of OspA₁₆₃₋₁₇₅ and self-peptides to 13 recombinant HLA-DRB molecules

MHC molecules are aligned on the X-axis according to their association with antibiotic-refractory or antibiotic-responsive Lyme arthritis (DRB1*1501/B5*0101 and DRB1*0701/B4*0101 are in linkage disequilibrium). MHC molecules were incubated with labeled peptides and MHC/labeled-peptide complexes were detected by fluorescence. The bars represent mean relative fluorescence of duplicate wells and the error bars denote the standard deviation. The amino acid sequence for each peptide is shown with core amino acids capitalized. Only one peptide (BBK32₃₉₂₋₄₀₄), in addition to OspA₁₆₅₋₁₇₃, had a strong antibiotic-refractory arthritis-associated DRB binding profile. All positive control peptides gave markedly positive results

(data not shown). The average fluorescence of the negative control peptides was below 30,000 units (data not shown).

Figure 2. Testing of the OspA₁₆₀₋₁₇₇, BBK32₃₉₂₋₄₀₄ and GK₂₉₅₋₃₀₇ peptides with Lyme arthritis patients' T cells

(A) T cell proliferation assays were performed using fresh PBMC from 11 Lyme arthritis patients and 13 normal control subjects. Patients' cells often recognized the OspA peptide, but they did not have reactivity above background with the BBK32 peptide. (B) Frozen cells from the same 11 patients tested in panel A, and frozen cells from 8 normal control subjects were stimulated with the GK peptide. While some patients had a proliferative response to the OspA peptide, none had a proliferative response above background to the GK peptide. For patient 3, the recorded proliferative response was 12,551 CPM. Peptides are denoted on the horizontal axis and the average CPM of duplicate wells on the vertical axis.

■ Number of peptide predicted to be bound by TEPITOPE / total number of peptide bound *in vitro* x 100
 ■ Number of peptide predicted to be bound by TEPITOPE / total number of peptide not bound *in vitro* x 100

Figure 3. Assessing the predictive power of TEPITOPE

The MHC/peptide binding results predicted by the algorithm were compared directly to the *in vitro* MHC/peptide binding results for 12 of the MHC molecules. True-positives identified by the algorithm were expressed as the percentage of the *in vitro* binding peptides that were predicted *in silico* using the threshold setting 3, 6 and 10 (black bars). The false-positive rate was expressed as the percentage of *in vitro* nonbinding peptides that were predicted *in silico* using the threshold settings of 3, 6 and 10 (grey bars).

Table 1
Borrelial proteins assessed for T cell epitopes associated with antibiotic-refractory Lyme arthritis

Known immunogenetic borrelial proteins		GenBank Accession Number
1.	Arthritis Related Protein (ARP)	<u>AAL25643</u>
2.	Borrelia Membrane Protein (BMPA)	<u>AAG00584</u>
3.	Decorin Binding Protein A (DBP A)	<u>NP_045697.1</u>
4.	Decorin Binding Protein B (DBP B)	<u>NP_045698</u>
5.	Fibronectin Binding Protein (BBK32)	<u>NP_212481</u>
6.	Outer Surface Protein A (OspA)	<u>NP_045688</u>
7.	Outer Surface Protein B (OspB)	<u>NP_045689.1</u>
8.	Outer Surface Protein C (OspC)	<u>AAA16058</u>
9.	Outer Surface Protein E (OspE)	<u>AAA22959.1</u>
10.	Outer Surface Protein F (OspF)	<u>AAA22960.1</u>
11.	Vmp-Like Sequence, Expressed (VlsE)	<u>AAC45733</u>
12.	p30 (Similar to Oligopeptide Permease)	<u>AAC44656</u>
13.	p37 (Flagellin A)	<u>NP_045619</u>
14.	p41 (Flagellin B)	<u>NP_212281</u>
15.	p58 (Oligopeptide ABC transporter)	<u>NP_212463</u>
16.	p66 (Outer Membrane Porin Protein)	<u>NP_2112737</u>
17.	p93 (Protoplasmic Cylinder Antigen)	<u>CAA49829.1</u>
Borrelial proteins with a peptide sequence similar to OspA ₁₆₅₋₁₇₃		
18.	Glycerol kinase (GK)	<u>NP_212375.1</u>
19.	Hypothetical protein BB0208	<u>NP_212342.1</u>
20.	Hypothetical protein BBG22	<u>NP_045482.1</u>
21.	Hypothetical protein BB0751	<u>NP_212885.1</u>

Table 2
MHC/peptide binding profiles of borrelial epitopes predicted by TEPITOPE

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Borrelial Peptides	MHC	Antibiotic-Refractory Associated						Antibiotic-Responsive Associated										
		0401	0101	0402	0405	0404	B5*0101	1501	0701	0102	0301	1101	1104					
Osp A																		
p8-16 ^a	I	G	L	I	L	A	L	I	A	2 ^b	1	2	2	4	1	6	1	1
p165-173	Y	V	L	E	G	T	L	T	A	1	1	2	3	4	3	7	1	2
p177-185	V	V	V	E	G	T	V	T	L	1	2	6	<10	3	2	7	<10	<10
BBK32																		
p394-402	Y	V	V	I	K	N	Q	K	N	1	1	1	9	4	5	<10	4	<10
Osp C																		
p14-22	F	L	F	I	S	C	N	N	S	1	1	3	8	4	1	6	1	5
OspE																		
p14-22	F	I	L	I	G	A	C	K	I	2	1	6	6	1	1	6	1	4
OspF																		
p5-13	M	F	I	I	C	A	I	F	A	2	1	2	2	2	1	3	1	1
BMPA																		
p111-119	Y	A	I	I	D	P	I	Y	S	1	1	4	7	3	3	3	1	1
DBP A																		
p15-23	L	T	I	L	V	N	L	L	I	1	1	1	1	2	1	9	2	2
DBP B																		
p14-22	F	F	K	L	L	V	A	C	S	1	3	6	<10	<10	5	7	1	2
p41																		
p1-9	M	I	I	N	H	N	T	S	A	1	2	1	<10	<10	2	7	8	8
p304-312	V	V	A	A	T	T	N	S	I	1	2	1	3	1	2	2	5	4
p58																		
p287-295	Y	F	Y	A	F	N	T	H	I	2	1	4	7	2	5	<10	9	<10
p295-303	I	K	P	L	D	N	V	K	I	2	1	3	3	4	1	9	7	6
p93																		
p620-628	V	V	S	E	S	N	F	E	I	1	2	4	2	2	2	8	<10	<10

^a Amino acid residues from the full length protein assessed for MHC binding using the TEPITOPE algorithm.

^b Threshold setting where MHC binding was predicted by TEPITOPE. Lower numbers correspond to a high stringency prediction (lower rate of false-positives), while lower numbers correspond to a low stringency prediction (higher rate of false-positives and lower rate of false-negatives). The value “<10” indicates MHC binding was not predicted at any TEPITOPE threshold setting.

MHC/peptide binding profiles predicted by TEPITOPE of borrelial epitopes with sequence homology to OspA₁₆₅₋₁₇₃

Table 3

Borrelial Peptides	MHC	Antibiotic-Refractory Associated						Antibiotic-Responsive Associated										
		0401	0101	0402	0405	0404	B5*0101	1501	0701	0102	0301	1101	1104					
Borrelial Peptides																		
Osp A																		
p165-173	Y	V	L	T	L	T	A	1	1	2	1	5	3	4	3	7	1	2
Glycerol Kinase	“	“	“	S	V	F	I	1	2	8	1	2	5	<10	3	4	8	<10
p297-305	“	“	I	“	I	“	K	1	2	2	1	1	1	9	5	5	1	3
Hypothetical Protein BB0208	“	“	P	“	“	“	“	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
p296-304	“	“	P	“	“	“	“	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Hypothetical Protein BBG22	K	T	“	“	“	“	“	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
p90-98	“	E	“	“	“	“	K	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Hypothetical Protein BB0751	T																	
p79-88																		

Table 4
Concordance between *in silico* and *in vitro* peptide/MHC binding for individual HLA-DR molecules^a

Threshold Range ^d	Number of peptides bound <i>in vitro</i> / Total number of peptides predicted to be bound by TEPITOPE (%) ^b		Number of peptides bound <i>in vitro</i> / Total number of peptides <u>not</u> predicted to bind by TEPITOPE ^c	
		≤ 3	≤ 10	>10
<u>Concordance</u>	<u>MHC</u>			
	0402	9/9 (100)	12/16 (75)	
HIGH	B5*0101	6/6 (100)	12/14 (86)	2/2
	0101	15/16 (94)	15/16 (94)	
	0404	14/15 (93)	14/16 (88)	
	0102	10/13 (77)	13/13 (100)	
INTERMEDIATE	0401	11/16 (69)	11/16 (69)	
	1501	5/8 (63)	9/15 (60)	0/1
	0405	8/13 (62)	9/16 (56)	
	1101	4/8 (50)	5/14 (36)	0/2
LOW	1104	3/6 (50)	5/11 (45)	1/5
	0701	3/9 (33)	5/14 (36)	1/2
	0301	1/3 (33)	3/14 (21)	0/2

^a Fifteen of the sixteen peptides were selected based on their prediction to be bound by DRB1*0101 and DRB1*0401 using a threshold range of 3. The only exception was GK297-306.

^b Frequency TEPITOPE correctly predicted *in vitro* peptide binding. Binding of the 16 peptides to each MHC molecule was assessed by TEPITOPE. Peptides predicted to bind were then evaluated for *in vitro* binding [bound *in vitro* / total predicted to be bound *in silico* (percents in parentheses)].

^c Frequency TEPITOPE failed to predict *in vitro* peptide binding. Peptides not predicted to bind MHC molecules by TEPITOPE, at any threshold setting, were evaluated for *in vitro* peptide binding [bound *in vitro* / total predicted not to be bound *in silico* (percents in parentheses)].

^d TEPITOPE threshold setting (stringency) used to predict binding.