HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2019 April 15.

Published in final edited form as:

J Immunol. 2018 April 15; 200(8): 2727–2737. doi:10.4049/jimmunol.1701764.

Epitope Binding Characteristics for Risk *versus* Protective DRB1 Alleles for Visceral Leishmaniasis

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Abstract

HLA-DRB1 is the major genetic risk factor for visceral leishmaniasis (VL). We used SNP2HLA to impute HLA DRB1 alleles and SNPTEST to carry out association analyses in 889 human cases and 977 controls from India. NetMHCIIpan2.1 was used to map epitopes and binding affinities across 49 *Leishmania* vaccine candidates, and across peptide epitopes captured from dendritic cells treated with crude *Leishmania* antigen and identified using mass spectrometry and alignment to a reference *Leishmania* genome. Cytokines were measured in peptide-stimulated whole blood from 26 cured VL cases and 8 endemic healthy controls. HLA-DRB1*1501 and DRB*1404/DRB1*1301 were the most significant protective versus risk alleles, respectively, with specific residues at amino acid positions 11 and 13 unique to protective alleles. We observed greater peptide promiscuity in sequence motifs for 9-mer core epitopes predicted to bind to risk (*1404/*1301) compared to protective (*1501) DRB1 alleles. There was a higher frequency of basic AAs in DRB1*1404-/*1301-specific epitopes, compared to hydrophobic and polar AAs in DRB1*1501-specific epitopes, at anchor residues P4 and P6 which interact with residues at DRB1 position 11 and 13. Cured VL patients made variable but robust interferon-γ, tumour necrosis factor and interleukin-10 responses to 20-mer peptides based on captured epitopes, with peptides

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Conflict of interest

The authors declare no conflicts of interest.

Statement about ethics

Ethical approvals for studies on Indian subjects were obtained from the ethical committee of the Institute of Medical Sciences, Banaras Hindu University (Varanasi, India). The study was carried out in accordance with the Declaration of Helsinki Principles, and each participant, or the parent/guardian of individuals <18 years old, signed informed consent forms to participate in the study and provide a blood sample.

Author contributions

Conceived and designed the experiments: TS MF NS JMB. Performed the experiments: TS JO NS APS AKrS. Analyzed the data: TS MF JMB. Supervised the clinical workup and experimental studies in patient donors and endemic healthy controls: JC. Wrote the paper: JMB with assistance from TS MF. Supervised the research in India: SS. Supervised the research in Australia and Cambridge: JMB. All authors read and approved the manuscript.

based on DRB1*1501-captured epitopes resulting in a higher proportion (odds ratio 2.23; 95%CI 1.17-4.25; P=0.017) of patients with interferon-γ:IL10 ratios >2-fold compared to peptides based on DRB1*1301-captured epitopes. Our data provides insight into molecular mechanisms underpinning the association of HLA DRB1 alleles with risk versus protection in VL in humans.

Introduction

The importance of host genetic factors in determining outcome of infection with *Leishmania* species causing visceral leishmaniasis (VL) is indicated by familial clustering (1) and high sibling risk ratios (2). In mice, different genes control innate versus adaptive immunity (reviewed (3)), but the MHC class II region (H-2) stands out as one of the earliest identified (4), with the clearest effects of any locus in murine models of complex infectious disease. This attests to the importance of CD4+ T cell mediated immunity in determining disease outcome in leishmaniasis, with polarized antigen-specific T helper 1 (Th1) and Th2/T regulatory responses affecting favourable versus adverse outcomes, respectively, in murine models (5) and human disease (6). Clinical VL caused by *L. donovani*, in particular, has been associated with high Th2/T_{reg} cytokine responses (6-10), while Th1-generated interferon-γ (IFN-γ) is higher in children infected with *L. infantum chagasi* that do not progress to clinical VL than in those who do (11). Nevertheless, HLA had not been highlighted as a putative susceptibility locus amongst a number of genome-wide linkage studies (12-15), and robust associations had not been observed in the few small studies in which HLA has been examined as a candidate gene region (reviewed (16)), likely because of low statistical power. As part of the Wellcome Trust Case Control Consortium 2 (WTCCC2), we undertook the first genome-wide association study (GWAS) of VL across major foci of disease caused by *L. donovani* in India and *L. infantum chagasi* in Brazil (17). Meta-analysis of discovery GWAS undertaken in 989 cases and 1089 controls from India and 357 cases in 308 families (1970 individuals) from Brazil identified the class II gene region HLA-DRB1-DQA1 as the major locus regulating susceptibility and resistance to clinical disease for *L. donovani* and *L. infantum chagasi* (Discovery Meta-P=1.6-×10^{-13}), with replication in a second sample of 941 cases and 990 controls from India (Combined P=2.76×10^{-17}, OR=1.41, 95%CI 1.30-1.52, across 3 cohorts). No other loci replicated at P<10^{-6} within India, and no non-MHC loci identified on the basis of the discovery GWAS in Indian samples were replicated in Brazil. The importance of this GWAS is the demonstration that HLA class II alleles are major genetic risk factors for VL that cross the epidemiological divides of geography and parasite species, renewing interest in research to understand the precise molecular basis to this and translate this knowledge to development of vaccines that will work even in genetically susceptible individuals.

Identification of peptides selected by MHC class II molecules during natural processing of proteins is fundamentally important to understanding the repertoire of CD4+ T cells regulating complex disease (18, 19). Improvements in mass spectrometry have facilitated examination of whole repertoires of peptides selected from natural processing, leading to key observations (reviewed (19)) on the nature of naturally processed peptides selected by class II: (i) variable in length (12-26 AA); (ii) families share a common 9 AA core; (iii) 9-mer binding core interacts with peptide binding groove, which consists of 4 major binding

pockets P1, P4, P6 and P9; (iv) most allelic differences between class II molecules reside within the binding pocket, which determine allele-specific binding motifs; (v) flanking residues increase peptide-binding affinity; and (vi) heterogeneity in flanking residues reflects enzyme specific proteolysis in antigen processing. This has underpinned development of predictive algorithms (20) to analyse epitope selection *in silico*, which can be compared with those determined experimentally. Here we use a combination of *in silico* tools and experimental epitope capture to characterize the epitopes that bind to risk versus protective DRB1 alleles for VL. We show greater peptide promiscuity and sequence motifs for 9-mer core epitopes predicted to bind to the peptide binding groove of risk (*1404/*1301) compared to protective (*1501) DRB1 alleles. We find that cured VL patients make robust IFN-γ, tumour necrosis factor (TNF) and interleukin-10 (IL-10) responses to 20-mer peptides based on protein sequences for captured epitopes, with peptides based on DRB1*1501-captured epitopes resulting in a higher proportion (odds ratio 2.23; 95%CI 1.17-4.25; P=0.017) of patients with interferon-γ:IL10 ratios >2-fold compared to peptides based on DRB1*1301-captured epitopes.

Materials and Methods

Study subjects

For HLA typing and imputation studies, we used subjects from the Indian discovery GWAS recruited from Bihar state in northeast India, as previously described (17). Cases and controls were matched for self-reported age, sex, religion, caste and geographic region of recruitment. The post-quality control sample used in the original GWAS was 989 cases and 1089 controls. For HLA imputation (cf. below) we used data for 889 unrelated cases and 977 unrelated controls. For whole blood assays we recruited 26 cured VL cases (14 females; 12 males; mean age 29±13 years, range 9 to 52 years) and 8 healthy endemic controls (1 female; 7 males; mean age 41±8 years, range 26 to 55 years) from the same study area as the GWAS. Of the cured cases, 23 received single dose Ambisome, and 3 received a 30-day course of Amphotericin B. All cases had achieved complete recovery. Samples were collected 4 to 7 months after completion of treatment. Dendritic cells (cf. below) were prepared from a cryobank of peripheral blood mononuclear cells for HLA-typed de-identified donors held at Lonza Biologics plc (Cambridge, UK).

Ethical considerations

Ethical approvals for studies on Indian subjects were obtained from the ethical committee of the Institute of Medical Sciences, Banaras Hindu University (Varanasi, India). The study was carried out in accordance with the Declaration of Helsinki Principles, and each participant, or the parent/guardian of individuals <18 years old, signed informed consent forms to participate in the study and provide a blood sample.

HLA imputation and association analysis

Imputation of classical HLA alleles at 2- and 4-digit level, as well as amino acid variants, in the Indian discovery GWAS case-control cohort was performed in SNP2HLA (21) using extensive reference data for the T1DGC (22) panel (10450 haplotypes). Frequentist association tests for each allele were performed in SNPTEST (23) under an additive model,

with the first 3 principal components for population genetic substructure as covariates. Odds ratios, associated *P*-values and frequency of the DRB1 alleles were determined.

In silico epitope predictions using NetMHCIIpan 2.1

We employed NetMHCIIpan 2.1 (20) to screen overlapping 20-mer peptides across 49 known vaccine candidate molecules, or across 12 proteins identified on the basis of epitope capture experiments (see results). This prediction tool outperforms other tools (24, 25) principally by implementation of NN-align, a neural network-based approach that combines the peptide sequence representation that was highly successful in predicting the binding specificity of HLA Class I molecules and now includes the representation of peptide flanking residues and peptide length applicable to HLA Class II molecules (25, 26). Binding affinities for each peptide are provided as nM IC_{50} values, with arbitrary cut-offs for strong and weak binding affinities set at IC_{50}, 1-50nM (=High) and 51-500nM (=Low). The y-axis of all binding affinity plots is represented as relative binding affinity expressed as $1\text{-}\log_{15,000}$ of the predicted nM binding affinity (20). Sequences for the 9-mer cores of 20-mer epitopes with binding affinities specific for risk or protective DRB1 alleles, or with binding affinity to both, were aligned using BlockLogo (27), and the resulting sequence motifs were visualized using WebLogo (28, 29). Peptides selected for whole blood assays are shown in Table S1 (cf. below). Note that reference to all binding affinities presented here are predicted, not measured, binding affinities.

Epitope capture from dendritic cells

Dendritic cells (DCs; $4\text{-}5\times10^6$) were prepared essentially as described (30) from cryopreserved peripheral blood mononuclear cells (PBMC) from human donors homozygous for risk (*1301, homozygous *1404 donors were not available) or protective (*1501) DRB1 alleles. Briefly, PBMC were isolated from fresh human whole blood by density gradient centrifugation in under 8 hours and stored in vapour phase nitrogen according to the ethically approved protocol and donors informed consent. To generate dendritic cells, cryopreserved PBMC were thawed and monocytes isolated using negative magnetic bead isolation (Stem Cell Technologies, Cambridge, UK). Monocytes were then differentiated into dendritic cells with human GM-CSF and human IL-4 (Peprotech, London, UK) in a tissue culture flask (BD Biosciences, Oxford, UK) for 5 days at 37°C, 5% CO_2. Crude *L. donovani* (strain MHOM/ET/67/HU3) antigen was prepared from stationary-phase promastigotes by resuspension in 5mM CaCl2, 10mM Tris, pH 7.4, 1:100 cOmplete™ EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, Castle Hill, Australia), and freeze-thawing 3x over liquid nitrogen (31). After differentiation the crude antigen was added to the dendritic cells to a final concentration of 50 μg/mL, along with TNFα and IL-1β for 24 hours to mature the dendritic cells. After maturation the DC were transferred to tubes and washed with assay medium before being centrifuged at 300g for 10 mins to pellet the cells. Once pelleted the medium was removed and the dendritic cell pellets stored at −80°C until HLA-DR purification. Membrane fractions were prepared by re-suspension and gentle homogenization of the cell pellets in hypotonic lysis buffer containing 0.5% IGEPAL CA-630 (octylphenoxypolyethoxyethanol; Sigma-Aldrich, Castle Hill, NSW, Australia), 50mM Tris-HCl (pH 8.0), 150mM NaCl, 2.5mM EDTA and protease inhibitors (Complete Protease Inhibitor Cocktail Tablet; Roche, Sydney, NSW, Australia) at ~10^7 cells/mL, and

rotated 1h at 4°C. Cellular debris was removed by ultracentrifugation at 180,000×g_{max} for 60min. Supernatants were retained, and the pellet resuspended and processed again through lysis buffer and ultracentrifugation. The combined clear supernatants were retained for purification of DRB1 molecules over CNBr-activated Sepharose 4 Fast Flow columns containing 5mg of L227 anti-human HLA-DR (1 mg antibody/mL of resin; L227 is specific for an HLA-DR monomorphic epitope – isotype IgG1) affinity matrix. The flow-through lysate was reloaded onto the Sepharose columns ×1. Columns were washed in 10 column volumes of buffer containing 50mM Tris, 150mM NaCl, and 0.5% IGEPAL CA-630 (final pH 8.0). Columns were sequentially washed by 5 column volumes of 50mM Tris and 150mM NaCl (final pH 8.0) followed by 5 column volumes of a high salt buffer, to remove non-specifically bound material (50mM Tris and 500mM NaCl (final pH 8.0)) and finally washed with 5 column volumes of 50mM Tris (pH 8.0). Bound HLA-DRB1*1301 and HLA-DRB1*1501 peptide complexes were eluted with 14mL of 10% acetic acid and eluates were collected as separate fractions of 2mL each. Western blotting was performed to determine (a) which fractions contained the eluted DRB1 molecules, and (b) to check for the ratio of αβ dimers to β-chain monomers indicative of the degree of dissociation of the DRB1 dimers and therefore of peptides from DRB1 molecules. DRB1-containing eluates were then filtered through 10kDa polyethersulfone ultracentrifugation filters (Sartorius, Dandenong South, Victoria, Australia) and frozen at −80°C. Samples were defrosted, 3.5mL H_2O added, vortexed, frozen with liquid N2, and frozen samples freeze dried ready for mass spectrometry. Mass spectrometry was performed by Proteomics International Laboratories Ltd (Perth, Western Australia). Samples were re-suspended in 100μL of 2% CAN/0.05% TFA and 50μL injected onto the HPLC system. Peptides were analyzed by LC-MS analysis using the Shimadzu Prominence nano HPLC system (Shimadzu, Malaga, Western Australia) coupled to a 5600 TripleTOF mass spectrometer (AB Sciex, Mulgrave, Victoria, Australia). Peptides were loaded onto an Agilent Zorbax 300SB-C18, 3.5μM (Agilent Technologies, Singapore) and separated with a gradient of 2-40% acetonitrile (0.1% formic acid) over 100min. Spectral data for the samples were analyzed against a database comprising the TriTrypDB-6.0_LdonovaniBPK282A1_ AnnotatedProteins with SwissProt curated Human protein sequences. Hits were considered as true hits against a *Leishmania* protein when the same spectrum matched with higher confidence to the *Leishmania* predicted peptide than to Human or Bovine Serum Albumin (BSA) matches for the same spectrum.

Whole blood assays

Whole blood was collected into heparinised tubes and samples were diluted 1 in 8 in serum-free complete medium comprising RPMI 1640 medium supplemented with 2 mM L-glutamine, 100μg/mL of streptomycin, and 100 IU/mL of penicillin (Gibco, USA). Diluted blood (180μL/well) was plated into 96-well U-bottomed plates (Nunc, Rochester, USA) and antigen added in triplicate wells at a final concentration of 10 μg/mL soluble *Leishmania* antigen (32) prepared from an Indian strain of *L. donovani*, 5 μg/mL individual peptides, 5 μg/mL PHA, or no antigen control. Peptides for 20-mer amino acid epitopes based on the predicted affinity of binding to risk and/or protective HLA DRB1 alleles (Table S1) were synthesized commercially (Peptide2.0, Chantilly, VA, USA). They were initially solubilized in dimethylsulfoxide (final concentration in the well <0.1% DMSO), diluted in endotoxin-free phosphate-buffered saline at a final concentration of 50 μg/mL, and stored at −80°C.

Cytokine release (IFN-γ, IL-10) was measured by ELISA in supernatants harvested at 24 h post antigen stimulation using matched antibody pairs (BD Pharmingen, Franklin Lakes, NJ, USA). The limit of detection for these ELISAs was 31 pg/mL. TNF was measured using the Human TNF-α ELISA MAX™ Set (Catalogue Number 430206; BioLegend, San Diego, CA, USA).

Statistical methods

Fisher's Exact Test was used to determine whether the proportions of responders to non-responders differed between peptides of different affinities for risk versus protective DRB1 alleles.

Results

Genetic associations of classical HLA DRB1 alleles with VL

A forest plot demonstrating the association between VL and imputed HLA DRB1 alleles at the 2- and 4-digit level is presented in Figure 1. This concurs with our previous analysis (17) showing that HLA DRB1*16, DRB1*01 and DRB1*15 allele groups provide significant protection (OR 0.43 to 0.79) from VL, while DRB1*14, DRB1*11, and DRB1*13 are significant risk (OR 1.37 to 1.76) groups. More specifically, for further functional analyses designed to relate DRB1 molecular function to VL susceptibility, we selected DRB1*1501 (OR 0.71; 95%CI 0.59-0.86; $P=3.01\times10^{-4}$) as representative of protective alleles, and DRB1*1404 (OR 1.37; 95%CI 1.09-1.71; $P=6.73\times10^{-3}$) or DRB1*1301 (OR 1.48; 95%CI 1.10-2.00; $P=0.01$) as representative risk alleles. These alleles were individually the most frequent risk and protective HLA-DRB1 alleles in the Indian population (HLA-DRB1*1301: 0.05; *1404: 0.09; *1501: 0.14). The analysis of imputed alleles also showed that VL susceptibility is associated with variants at amino acid (AA) positions 9, 11, 13, and 37 (Figure 2) which contribute to forming the DRB1 binding groove pockets that interact with residues of the 9-mer core of the peptide (33) (Figure 3). For example, Proline and Leucine (pocket P6 of the binding groove) at DRB1 AA position 11, and Serine at AA position 37 (pocket P9 of the binding groove), are associated with protection (OR 0.67; 95%CI 0.58-0.77; $P=1.48\times10^{-8}$) and are unique to the protective allele groups DRB1*01/*15/*16. Alternative AA alleles (Serine, Valine, Asparagine) at DRB1 AA position 11 are associated with risk (OR 1.45; 95%CI 1.28-1.65; $P=1.27\times10^{-8}$) and are common to risk allele groups DRB1*03/*04/*09/*10/*11/*12/*13/*14. This evidence highlighting AA positions that influence structure and conformation of the peptide-binding groove of the DRB1 molecule provided the rationale for further functional analysis of epitope binding affinities.

In silico epitope predictions for candidate Leishmania vaccines

To determine whether risk versus protective 4-digit HLA-DRB1 alleles differ in epitope binding affinities and peptide repertoire for *Leishmania* antigens we selected a subset of 49 known diagnostic and vaccine candidate proteins for *in silico* analysis (Table I). Using NetMHCIIpan 2.1 (20), a sliding window of 1-mer overlapping 20-mer peptides based on the full-length amino acid sequences of the 49 antigens were screened in order to identify peptides with strong and weak binding affinities (arbitrarily IC_{50}, 1-50nM=strong; 51-500nM=weak). Figure S1 provides plots mapping binding affinities for epitopes across

Leishmania proteins predicted to bind to risk DRB1*1404 compared to protective DRB1*1501 4-digit alleles. Of interest, some well-studied vaccine candidates (40, 59) were almost devoid of epitopes that would be predicted to bind to risk or protective DRB1 alleles (see, for example, A2 and HASPB1 in Figure S1). To gain a better understanding of the nature of epitopes binding to different DRB1 alleles, we selected the 9-mer core for the 20-mer with the top binding affinity to either HLA-DRB1*1404 or HLA-DRB1*1501, or with equal binding affinity to both. For the analysis of DRB1*1404- versus DRB1*1501-specific epitopes, preferential binders were defined as those with ≤500nM affinity for one DRB1 allele and ≥1000nM for the alternative allele. Table S1 gives examples of the NetMHCIIpan 2.1 output and how these 9-mer cores were selected. Across all proteins, the total number of unique epitopes with preferential binding to DRB1*1404 was 97 (mean±SD binding affinity for *1404 = 350±93; for *1501 = 1231±260), with 82 unique epitopes preferentially binding to DRB1*1501 (mean±SD binding affinity for *1501 = 356±105; for *1404 = 1277±469). There were 188 unique epitopes with peak binding affinities <100nM common to both DRB1 alleles. WebLogo plots (Figure 4) show that the sequence motif for 9-mer core peptides that bind preferentially to DRB1*1501 differs from those of DRB1*1404-preferential or common binders. In addition to greater promiscuity of amino acid usage in DRB1*1404-preferential 9-mer core peptides, the sequence motif plots show that the anchor residues interacting with the HLA-DR binding pockets 4 (P4) and P6 differ between the two alleles in terms of the nature and frequencies of amino acids at these positions. While hydrophobic residues are generally preferred for P1 for both alleles, P4 preferred residues are polar-hydrophilic (specifically, serine and threonine) or basic for DRB1*1404 but mainly non-polar hydrophilic (specifically, glycine) or hydrophobic in DRB1*1501. Similarly, residues that interact with P6 of the HLA-DR molecule are generally basic or polar-hydrophilic (specifically, serine and threonine) for the risk DRB1*1404 allele but non-polar hydrophilic (specifically, glycine) or hydrophobic for the protective DRB1*1501 allele. These differences are of specific interest given that the anchor residues 4 and 6 interact with the HLA-DRB1 residues at positions 11 and 13, respectively (Figure 3). The latter are unique to the protective HLA-DRB1*15 allele groups that our HLA imputation studies demonstrate are associated with VL (Figure 2). Except for residues at position 1, which are similarly conserved across all 9-mer core peptides, motifs of 9-mer core peptides that are common to both DRB1 alleles (Figure 4) exhibit a diverse sequence pattern with lower sequence conservation at P4 and P6 compared to allele-specific motifs. The differences in amino acid usage for motifs across the 20-mer peptides from which 9-mer cores were selected also reflect the differences apparent in the 9-mer core epitope motifs (Figure S2).

Epitope capture from dendritic cells

In silico analysis provided evidence for different characteristics of peptide motifs binding to 9-mer cores for epitopes predicted to bind to risk versus protective DRB1 4-digit alleles. To determine whether similar differences occur for naturally processed and presented leishmanial antigens, we eluted peptides from DRB1 molecules purified from dendritic cells prepared from donors homozygous for risk (DRB1*1301) or protective (DRB1*1501) 4-digit alleles. In this experiment, 428 unique peptides were captured from DRB1*1501 human dendritic cells and 482 from DRB1*1301. Of these, 12 peptides (range 8- to 28-mers) mapped definitively back to the *Leishmania* genome alone, one in common between

DRB1*1301 and DRB1*1501 dendritic cells, 6-specific to DRB1*1301, and 5 specific to DRB1*1501 (Figure 5). One peptide (not shown) had identical spectral matches to elongation factor 1-alpha against both human and *Leishmania* databases. This could represent a genuine auto-antigen or could be a captured *Leishmania* epitope.

In silico epitope binding affinity predictions for captured Leishmania antigens

As for diagnostic/vaccine antigens, NetMHCIIpan2.1 was used to map epitopes across the full-length amino acid sequences for *Leishmania* proteins (Figure 5) from which epitopes had been captured. Figure S3 provides epitope binding affinity plots for 1-mer overlapping 20-mers predicted to bind to risk DRB1*1301 compared to protective DRB1*1501 4-digit alleles, with arrows indicating the AA positions matching the captured epitopes. Proteins from which epitopes were captured were generally large (Figure 5; 363AA to 3790AA in length), with multiple epitopes predicted to bind to one or both DRB1 alleles. Importantly, peak binding 20-mers incorporating captured peptides for all except one eluted peptide (from hypothetical protein LdBPK_331570.1; Table S1) were predicted to bind at <500nM, generally with stronger predicted binding affinity for the DRB1 allele from which they were captured. Based on 9-mer cores for the 20-mer (incorporating the captured peptides) with the strongest DRB1 allele-specific binding affinity (Figure 6) there was, as in Figure 4, a bias towards hydrophobic and polar AAs in DRB1*1501-specific peptides at 4 and 6, pointing again to the potential importance of these anchor residues interacting with the DRB1 residues at position 11 and 13 (Figure 3), shown to be associated with VL (Figure 2).

Cytokine responses to peptides based on captured epitopes

To determine whether epitopes captured from dendritic cells treated with crude *Leishmania* antigen *in vitro* were representative of antigens processed and presented to naturally exposed individuals, we had peptides synthesized based on captured epitopes (Table S1) for use in whole blood assays (60, 61). The peptides were based on the 20-mer with the strongest predicted binding that overlapped the captured epitopes. Cytokine responses to the 12 peptides were studied in cured patients and endemic healthy controls. Analysis focused on comparing cytokine responses for peptides of differing binding affinities for risk versus protective alleles. Cured VL patients made robust but individually variable IFN-γ, and IL-10 responses to 20-mer peptides based on captured epitopes (Figure 7). Of interest, peptides based on DRB1*1501-captured epitopes resulted in a higher proportion (odds ratio 2.23; 95%CI 1.17-4.25; P=0.017) of patients with IFN-γ:IL10 ratios >2-fold compared to peptides based on DRB1*1301-captured epitopes (Figure 7D). TNF measured in a subset of cured patients (Figure 8A) again showed robust but individually variable responses across all peptides based on captured epitopes. Figure 8 (B to D) shows plots of cytokine responses to all 12 peptides across endemic healthy controls. Very few endemic healthy controls showed IL-10 or TNF responses to peptides or SLA. Individual IFN-γ responsiveness to peptides or SLA among these individuals can be accounted for by endemic exposure to the parasite, as asymptomatic infections based on modified quantiferon positivity (61) are common in the population (62).

DISCUSSION

Previously we identified the class II gene region HLA-DRB1-DQA1 as the major locus regulating susceptibility and resistance to clinical disease for *L. donovani* in India and *L. infantum chagasi* in Brazil (17), indicating that HLA class II alleles provide genetic risk factors for VL that cross the epidemiological divides of geography and parasite species. Here we used HLA imputation to further refine genetic risk factors for VL in India at the level of 4-digit classical HLA-DRB1 alleles. We also demonstrate that the association maps to specific amino acids encoded in exon 2 that determine the interaction with amino acids at positions P4 and P6 of the 9-mer cores of foreign epitopes binding to the groove created by DRA1/DRB1 alpha/beta dimers. Based on these findings we used *in silico* analysis of both known *Leishmania* vaccine candidate antigens, as well as epitopes captured from dendritic cells of homozygous HLA DRB1 donors treated with crude *Leishmania* antigen, to demonstrate major differences in sequence motifs for 9-mer cores of *Leishmania* epitopes that bind preferentially to risk compared to those that bind preferentially to protective DRB1 alleles. Further, individuals drug-cured from VL showed strong recall T cell responses to peptides based on captured epitopes, indicating that these captured epitopes were representative of antigens processed and presented to antigen-specific CD4 T cells in naturally exposed individuals.

Immunological studies have long indicated the importance of CD4+ T cell mediated immunity in determining disease outcome in leishmaniasis, with polarized antigen-specific T helper 1 (Th1) and Th2/T regulatory responses affecting favourable versus adverse outcomes, respectively, in murine models (5) and human disease (6). The question of how the MHC affects the differentiation of Th1 compared to Th2/T regulatory populations in the context of a complex infection has been a long-term interest of immunologists (63-65). Early work demonstrating that peptides of highest affinity for a given MHC class II molecule elicited a shift towards the Th1 subset [e.g. (66)], led to theories of immuno-dominance of pathogen molecules and a role for affinity (i.e. the strength of a single bond) or avidity (i.e. the combined strength of multiple bond interactions) in dictating Th1 versus Th2 immunity (63). In support of this, specific HLA class II genotypes have been linked to Th1- or Th2-like responses against defined antigens in malaria and leprosy (64, 65). A growing body of studies in human and avian models of infection has explored how peptide promiscuity and sequence motif relates to functional avidity and disease susceptibility (67-71). Peptide-binding repertoires differ across MHC class I and II alleles (70, 71) with the structure of the binding groove influencing peptide-binding promiscuity and explaining resistance to viral infection (67). In humans, HLA-DRB1 variants linked with low viremia in HIV promiscuously present a larger breadth of peptides with lower functional avidity when compared to DRB1 variants linked with high viremia (71). Similarly, dominant and highly promiscuous epitopes characterize the CD4+ T helper cell response of spontaneously controlled Hepatitis C virus infection (69). We therefore hypothesised that sequence motifs of peptides predicted to bind preferentially to protective versus risk alleles could highlight important residues and help to explain differences in $CD4^+$ T cell cytokine responses. When we tested this in 49 known vaccine candidates we observed differences in sequence motifs and greater promiscuity of peptide motifs for 9-mer core epitopes that were predicted to bind

to the risk alleles DRB1*1404 compared to the strongest protective DRB1*1501 allele. This perhaps seems counter-intuitive relative to the earlier HIV (71) and hepatitis (69) studies. However, strong pro-inflammatory responses characteristic of low viremia in the latter cases may contribute to disease pathogenesis in clinical VL (72). Ultimately it is the balance between macrophage-activating Th1 cytokines IFN-γ/TNF and regulatory IL-10 that is important in determining a curative response, and is also a hallmark of vaccine-induced cure in pre-clinical models of disease (45). The critical question in our study was whether the same pattern of differences between sequence motifs for the 9-mer cores of epitopes binding to risk versus protective DRB1 alleles would emerge if naturally processed and presented antigens were identified and studied in endemic human disease cases. Our study of epitopes captured from dendritic cells indicated that this was, indeed, the case.

To reflect the functional role of cytokine responses in determining disease outcome we examined IFN-γ, TNF and IL-10 responses to peptides based on captured epitopes in drug-cured VL patients and endemic healthy controls. Robust but variable responses for all three cytokines were observed across all peptides, indicating that these captured epitopes were representative of antigens processed and presented during natural infections. Of note, there were no obvious differences in cytokine responses related to whether peptides were predicted to bind with strong or weak affinity to the risk versus protective DRB1 alleles from which epitopes were captured. However, as to be expected given the strong association between VL and HLA-DRB1 (17), only 3 of 26 cases studied were heterozygous for the single nucleotide polymorphic variant that tags the protective DRB1*1501 allele (data not shown). Hence, it was not possible to differentiate cured case donors as carrying risk versus protective alleles when studying immune responses to peptides based on captured antigens. Nevertheless, it was of interest that there was a bias towards higher IFN-γ:IL-10 ratios in cured patients in response to peptides based on antigens eluted from the dendritic cell donor homozygous for the protective DRB1*1501 allele. Further in-depth analysis of responses to these captured antigens in asympotomatic individuals identified as quantiferon-positive (61) in endemic areas will help to determine whether these specific antigens preferentially constitute useful candidates for future vaccines.

In summary, our study has refined the association between HLA Class II molecules and susceptibility to VL in this Indian population, and demonstrated clear differences in sequence motifs for 9-mer cores epitopes that bind to risk versus protective DRB1 alleles. These findings contribute to further understanding of the molecular interaction between DRB1 and CD4 T cells that determine the outcome of VL disease caused by *L. donovani*, providing novel naturally processed and presented candidate antigens that could contribute to future vaccine design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the hospital staffs at Kala–azar Medical Research Centre, Muzaffarpur for their assistance in the collection of blood samples and all research scholars of Infectious Disease Research Laboratory, Banaras Hindu University for their kind help during the study.

This work was supported by the NIH as part of a Tropical Medicine Research Centre award.

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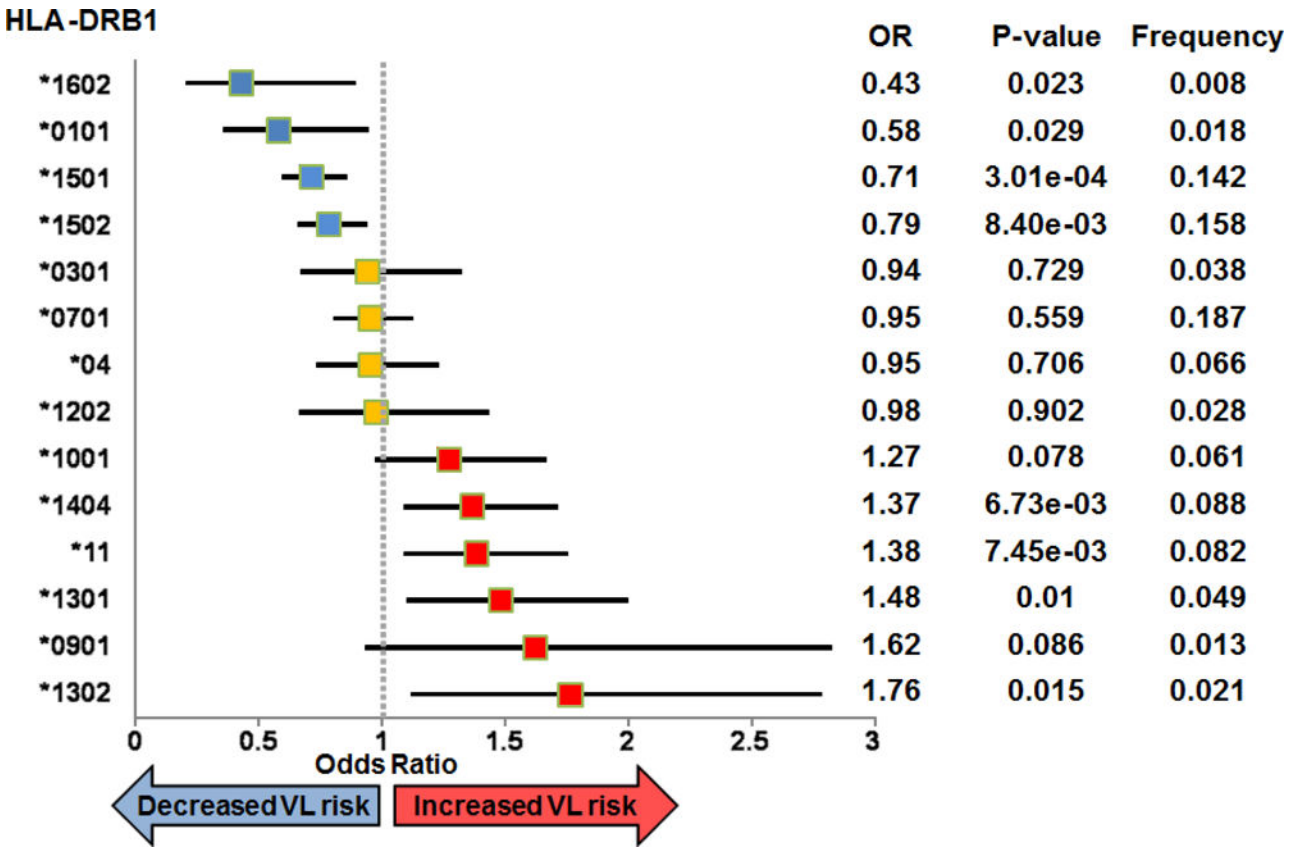

FIGURE 1.

Forest plot showing associations between VL and imputed 2- or 4-digit classical HLA DRB1 alleles. The plot shows odds ratios (OR) and 95% confidence intervals for risk (OR>1; red symbols), neutral (OR~1; yellow symbols), and protective (OR<1; blue symbols) haplotypes. Information to the right of the plot shows values for the OR, the P-value for the association, and the allele frequency.

Mature protein position	Amino acid code	Amino acid	Peptide pocket	Amino acid frequency	DRB1 allele groups	P-value of disease association	Odds ratio	95% confidence interval
9	W	Trp	9	0.48	*01/*07/*15/*16	1.27E-08	0.69	0.61-0.78
11	PL	Pro/Leu	6	0.33	***01/*15/*16**	1.48E-08	0.67	0.58-0.77
11	SVD	Ser/Val/Asp	6	0.48	*03/*04/*09/*10/ *11/*12/*13/*14	1.27E-08	1.45	1.28-1.65
13	SFG	Ser/Phe/Gly	4	0.44	*01/*03/*09/*10/ *11/*12/*13/*14	7.36E-08	1.43	1.25-1.62
37	S	Ser	9	0.33	***01/*15/*16**	1.48E-08	0.67	0.58-0.77
37	SL	Ser/Leu	9	0.36	*01/*12/*15/*16	3.30E-08	0.68	0.6-0.78

FIGURE 2.

SNPTEST results for association between Indian VL and imputed AA variants within the HLA-DRB1 binding groove. Imputation was carried out using SNP2HLA and reference data from T1DGC, as referenced in the main text. Dark grey shading indicates protective alleles (odds ratio <1), light grey shading risk alleles (odd ratio >1). Bold lettering highlights AA at positions 11 and 37 that are unique to protective allele groups, as discussed in the text.

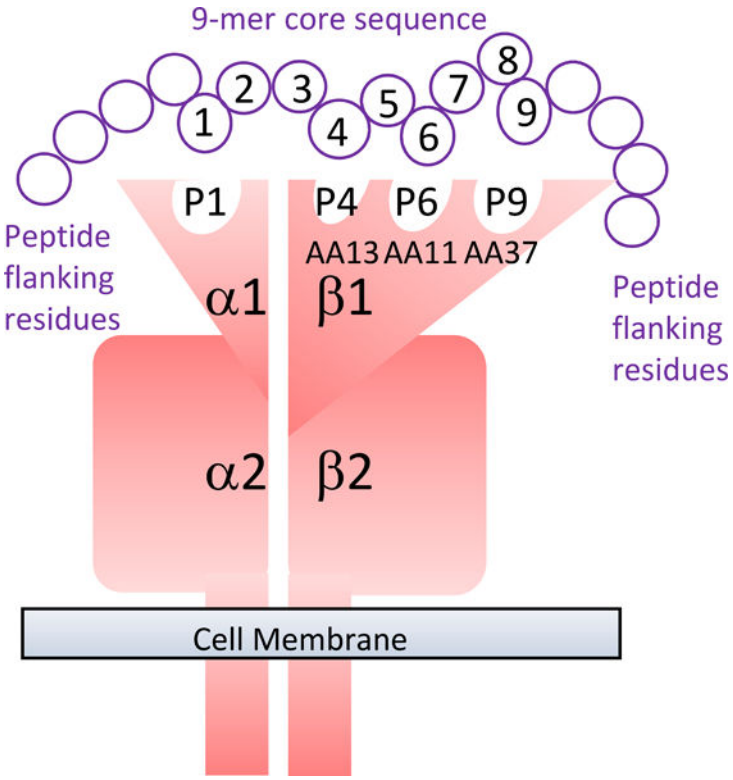

FIGURE 3.

Diagrammatic representation of the DRA/DRB alpha/beta dimer to show how specific amino acids in the binding groove of DRB1 form the pockets that interact with different positions in the 9-mer core epitope of the antigen being presented.

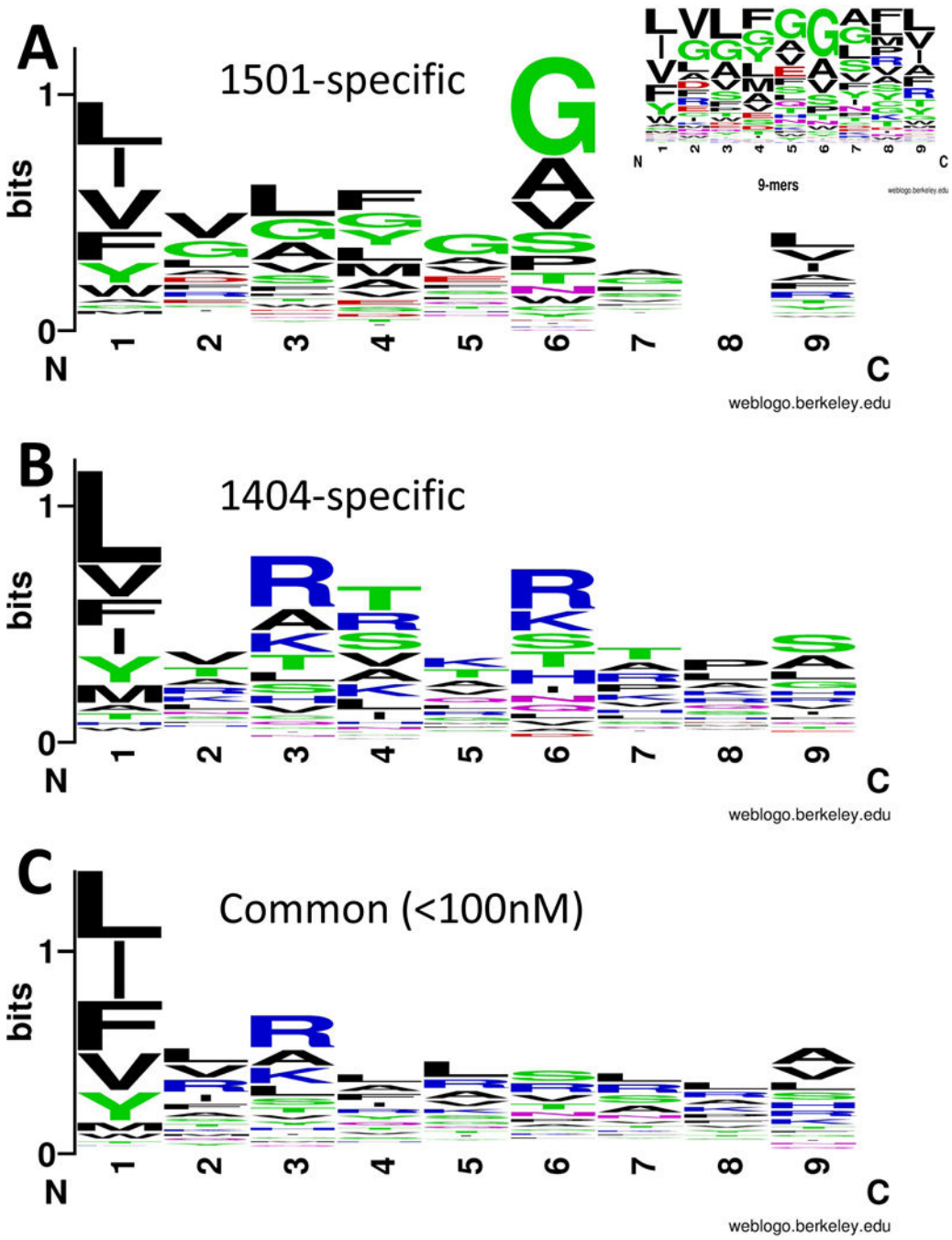

FIGURE 4.

Sequence motifs that characterise 9-mer core epitopes for 20-mer peptides predicted to bind preferentially to (A) DRB1*1501 or (B) DRB1*1404 alleles, or (C) predicted to bind to both DRB1 alleles at <100nM. Preferential binders were defined as those with ≤500nM affinity for one DRB1 allele and ≥1000nM for the alternative allele (see Table S1 for examples). *In silico* predictions for 20-mer peptides were generated across 49 *Leishmania* vaccine candidate proteins using NetMHCIIpan 2.1. Sequences were aligned using BlockLogo and plotted using WebLogo, as referenced in the main text. The plots are based on 9-mer cores

for 20-mer epitopes that spanned the peaks as plotted in Figure S1. Only one 9-mer core was selected to represent each epitope peak. The colours of the amino acids (AA) correspond to their chemical properties: polar AA are shown in green (G,S,T,Y,C; note G is non-polar when it forms a bond with other AA) or purple (Q,N); basic AA in blue (K,R,H); acidic AA in red (D,E); hydrophobic AA in black (A,V,L,I,P,W,F,M). The size of the letter relates to the frequency with which the AA is found in this position of core 9-mers, the overall peak height on the y-axis indicates the degree of conservation for specific epitopes at this location. Position 8 for DRB1*1501 showed no AA conservation; frequencies of AA at this position are shown in the insert figure.

Leishmania protein	ID	Peptide	Peptide Length	Protein Length
Elongation factor 1-α	LdBPK_170170.1	IGGIGTVPVGRVE	13	449
Mitogen-activated protein kinase	LdBPK_191480.1	SSEAKAFILSQPRR	14	363
Hypothetical protein	LdBPK_050430.1	GFTIIDAD	8	675
Hypothetical protein	LdBPK_100800.1	GELLKLPQ	8	1988
PIF1 helicase	LdBPK_110320.1	RANNINLP	8	1514
Protein kinase	LdBPK_210190.1	LQSAATHTDSI	11	1711
Hypothetical protein	LdBPK_171350.1	ILLVGDRAKDQVVNLHLMI	19	1065
Hypothetical protein	LdBPK_351710.1	SNVGVCSRVGVARLWF	16	444
ATP-dependent DNA helicase	LdBPK_090640.1	GAGSGKTQTMAAR	13	996
Hypothetical protein	LdBPK_331570.1	SPPRVVTAATAPVGSPTAAATAFSGSAP	28	3442
Hypothetical protein	LdBPK_302400.1	AGFIALTAR	9	3790
Inositol 1,4,5-trisphosphate receptor	LdBPK_160290.1	LLAGCTLLQ	9	2874

FIGURE 5.

Leishmania peptides eluted from human dendritic cells treated with crude *Leishmania* antigen. ID, is for the matching proteins in the TriTrypDB-6.0_LdonovaniBPK282A1_AnnotatedProteins database. Light grey shading indicates epitopes captured from the risk DRB1*1301 Class II molecules, dark grey shading from the protective DRB1*1501. No shading indicates the epitope common to both allele groups. Mean length of proteins = 1625AA.

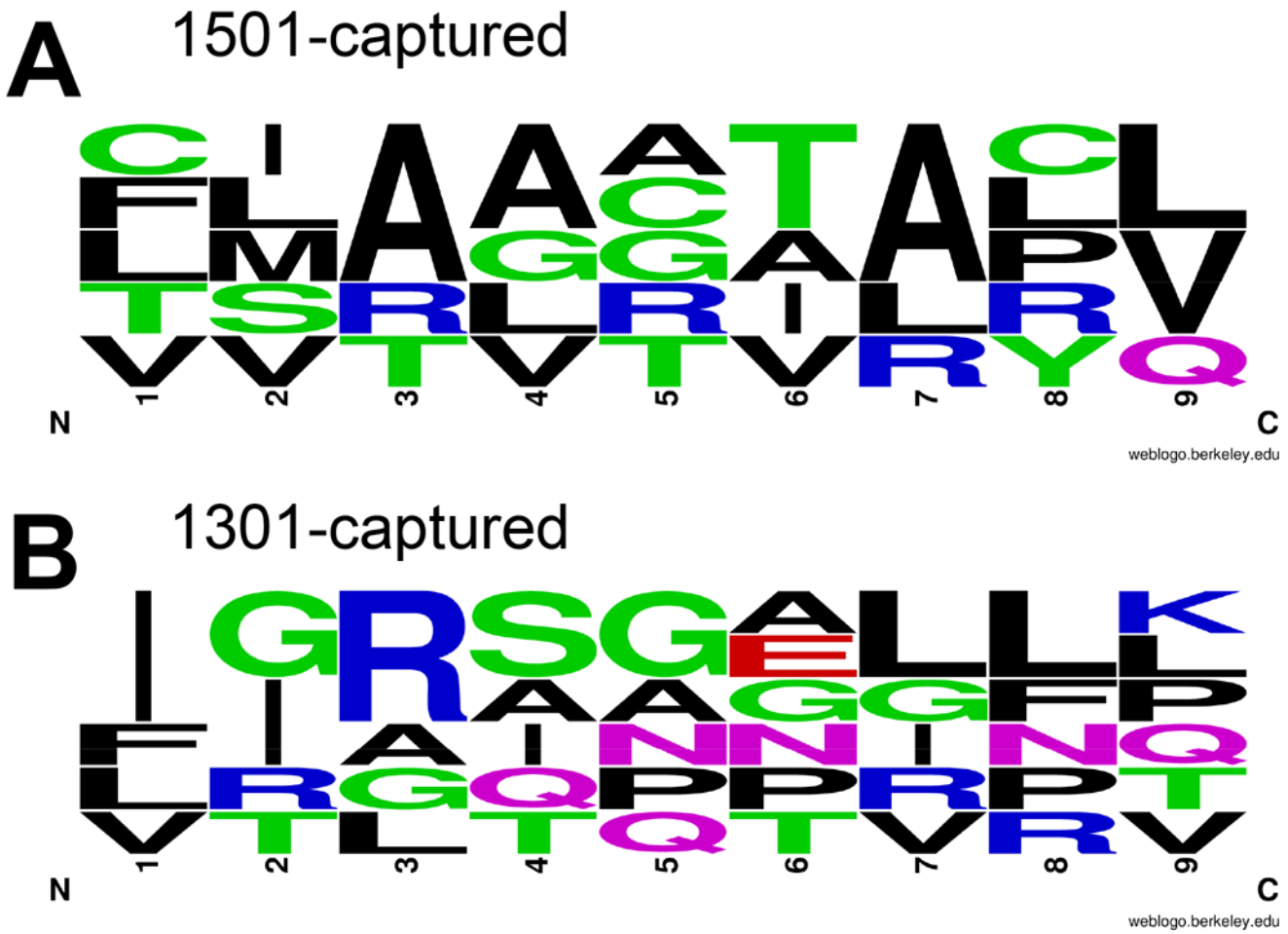

FIGURE 6.

Sequence motifs that characterise 9-mer core epitopes for 20-mer peptides incorporating peptide sequences as captured from dendritic cells from donors homozygous for risk (*1301) or protective (*1501) DRB1 alleles. Plots generated as in Figure 2, except that only the relative frequencies of AA are shown at each location in the 9-mer core of the 20-mer epitope since there were too few captured epitopes to determine degree of sequence conservation.

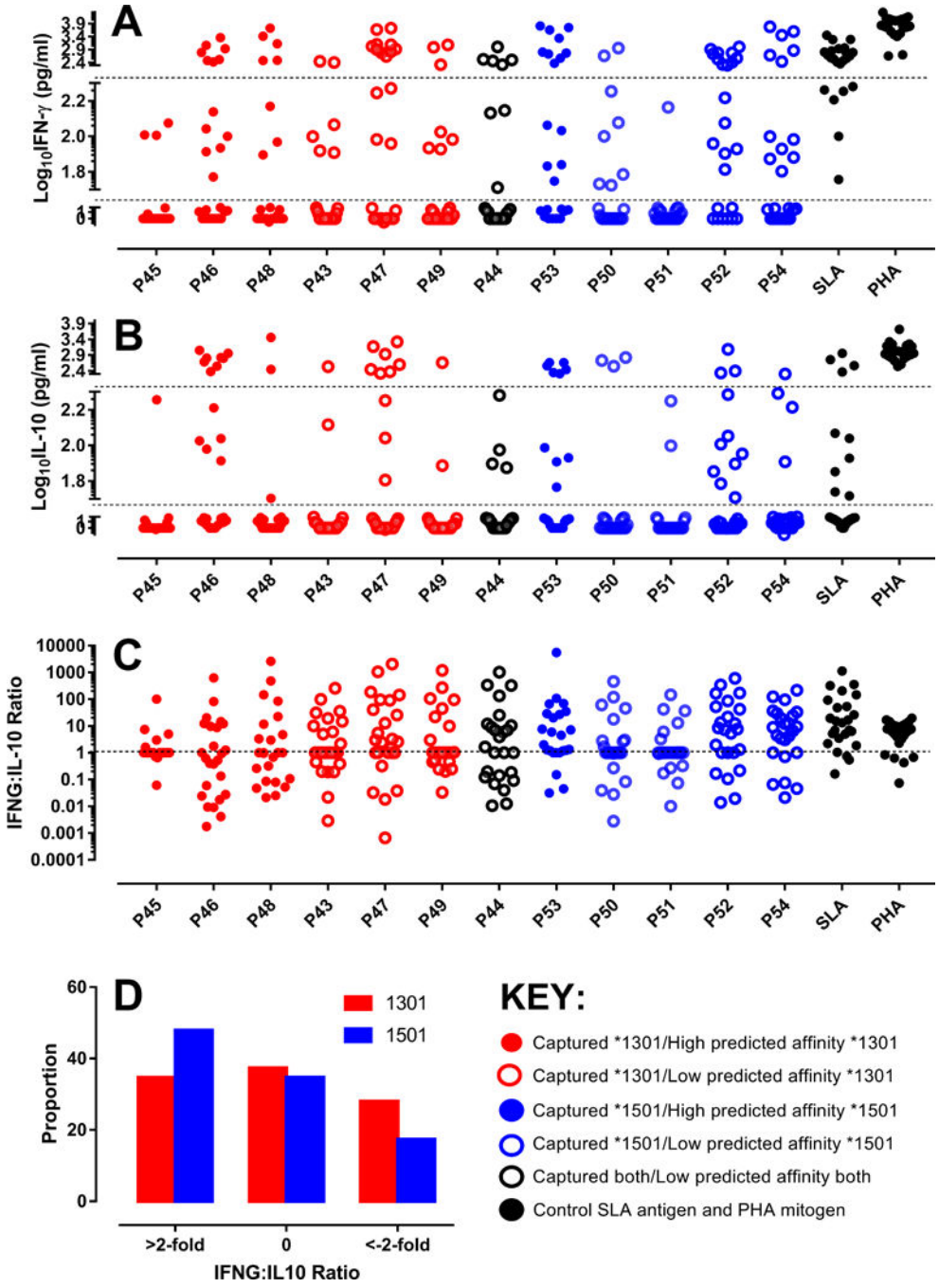

FIGURE 7.

(A) IFN-γ and (B) IL-10 responses in cured patients. The dotted lines represent cut-offs for high-responders (>200 pg/ml), responders (50-200 pg/ml) and non-responders (<50 pg/ml). All values are after subtraction of the NIL antigen control. The y-axes are split to give visual emphasis to the cut-offs for responder status. They are numerically contiguous; all values appear on the graph. TNF responses measured in a subset of these individuals are presented in Figure 5. (C) provides IFN-γ:IL-10 ratios. (D) shows the proportions of individuals with IFN-γ:IL-10 ratios >2-fold, between −2-fold and +2-fold, and <−2-fold. The key indicates

which DRB1 allele peptides were captured from, and what their predicted affinity of binding was to that DRB1 allele.

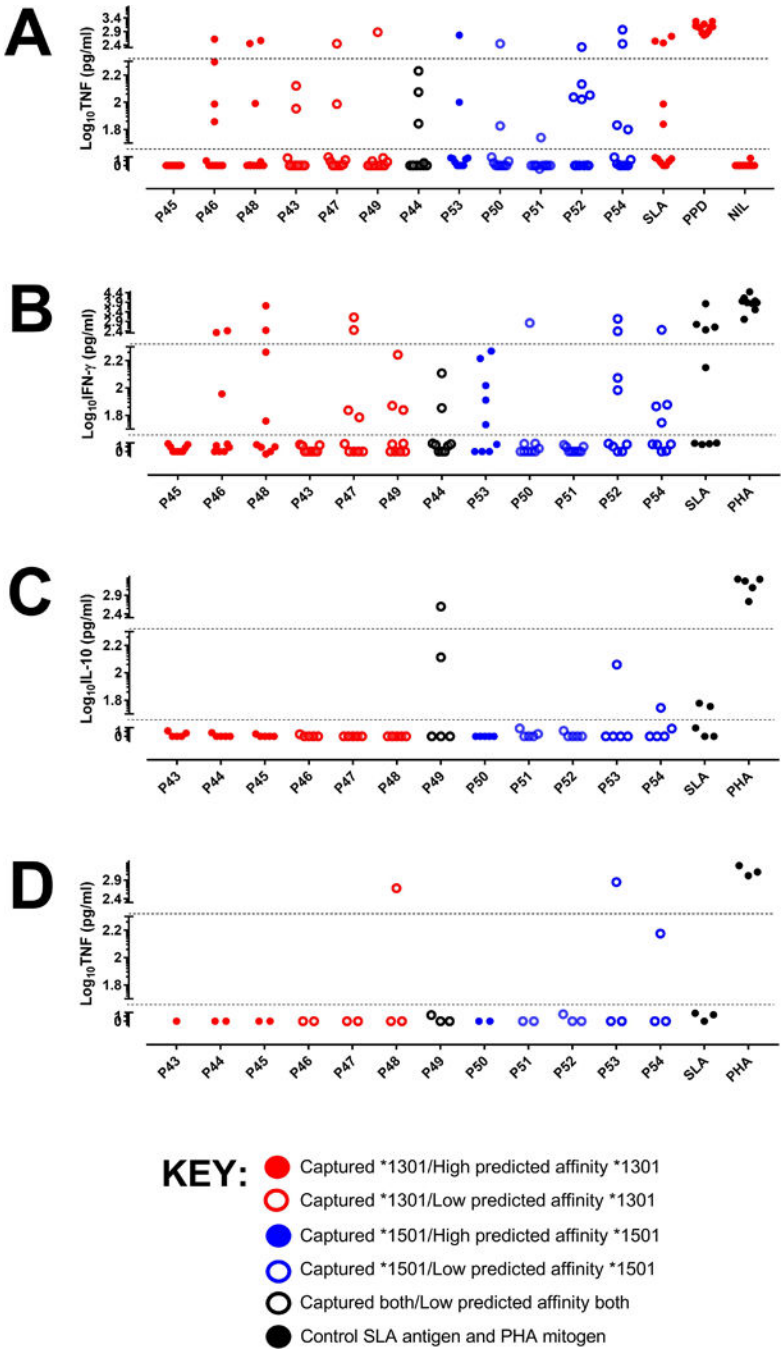

FIGURE 8.

(A) TNF responses for a subset of cured cases for whom IFN-γ and IL-10 were measured (see Figure 4). (B), (C) and (D) are cytokine responses in endemic healthy control donors; (B) IFN-γ, (C) IL-10, and (D) TNF. The dotted lines represent cut-offs for high-responders (>200 pg/ml), responders (50-200 pg/ml) and non-responders (<50 pg/ml). All values are after subtraction of the NIL antigen control. The y-axes are split to give visual emphasis to

the cut-offs for responder status. They are numerically contiguous; all values appear on the graph.

Table I

Details of 49 proteins selected for *in silico* analysis of epitope binding affinity predictions using NetMHCIIpan2.1

Protein name	Product	GeneDB/GenBank Accession	Reference
A2 LINJ220670	hypothetical protein	UniProtKB - A4HZU7_LEIIN	(34)
CPL2	cathepsin L-like protease	LdBPK_080960.1	(35)
CPA/CBA	cysteine peptidase A	LdBPK_191460.1	(36)
CPB	cysteine peptidase B	LdBPK_070600.1	(36)
CPC	cysteine peptidase C	LdBPK_290860.1	(37)
gp46/PSA/M-2	surface membrane protein gp46	LdBPK_311490.1	(38)
gp63/MSP	GP63, leishmanolysin	GenBank: ACT31401.1	(39)
HASPB1/K26	hydrophilic acylated surface protein B	GenBank: CAA09789.1	(40)
HbR	hemoglobin receptor	LdBPK_210300.1;	(41)
Hsp70	HSP70-like protein	LdBPK_283000.1	(39)
J41	hypothetical protein	LdBPK_331620.1	(42)
J89	hypothetical protein	LdBPK_260990.1	(42)
KMP11	kinetoplastid membrane prot-1	LdBPK_352260.1	(43)
L15	anaphase promoting complex subunit	LdBPK_354570.1	(42)
L21	nucleolar protein family a member	LdBPK_343810.1	(42)
lmd29	hypothetical protein	LdBPK_180600.1	(42)
L302.06	hypothetical protein, conserved	LdBPK_040220.1	(42)
L31/584C	60S ribosomal subunit L31	LdBPK_353330.1	(42)
L37	60S ribosomal L37	LdBPK_342620.1	(42)
L5	60S ribosomal L5	LdBPK_351870.1	(44)
L3	ribosomal protein L3-like	LdBPK_323320.1	(44)
LACK/p36	activated protein kinase c receptor	LdBPK_282970.1	(45)
LeIF	eukaryotic initiation factor 4a	LdBPK_010790.1	(46)
Lepp12	phosphoprotein lepp12	LdBPK_365970.1	(47)
LmSTI1	stress-induced protein sti1	LdBPK_081020.1	(48)
LPG3	lipophosphoglycan biosynthetic	LdBPK_290790.1	(49)
M18	amastin-like protein	LdBPK_080720.1	(42)
N52	vacuolar ATP synthase subunit	LdBPK_120480.1	(42)
NH36	nonspecific nucleoside hydrolase	LdBPK_181570.1	(50)
PABP1	polyadenylate-binding protein 1	LdBPK_355360.1	(51)
PABP2	polyadenylate-binding protein 2	LdBPK_354200.1	(51)
PABP3	poly(A)-binding protein, putative	LdBPK_250080.1	(51)
Pxn4	peroxidoxin_4	LdBPK_230050.1	(52)
PO	60S acidic ribosomal protein P0	LdBPK_072480.1	(53)
P25	hypothetical protein	LdBPK_354800.1	(42)
P31	hypothetical protein, conserved	LdBPK_342300.1	(42)
PRP-2	paraflagellar rod protein 2C	LdBPK_161510.1	(54)
Q24	dynein light chain, flagellar outer arm	LdBPK_320240.1	(42)

Protein name	Product	GeneDB/GenBank Accession	Reference
Q51	hypothetical protein	LdBPK_301750.1	(42)
R32	hypothetical protein	LdBPK_333110.1	(42)
R71	60S ribosomal protein L22	LdBPK_363430.1	(42)
S4	40S ribosomal protein S4	LdBPK_131120.1	(55)
S33	eukaryotic initiation factor 5a	LdBPK_250740.1	(42)
SOD B1	iron superoxide dismutase	LdBPK_321910.1	(56)
SMT	sterol 24-c-methyltransferase	LdBPK_362520.1	(57)
TSA/TRYP	tryparedoxin peroxidase	LdBPK_151140.1	(31)
TUZIN1	Tuzin, putative (62.3 kDa)	LdBPK_342640.1	(58)
TUZIN2	Tuzin, putative (58.7 kDa)	LdBPK_080730.1	(58)
TUZIN3	Tuzin, putative (70.2 kDa)	LdBPK_080740.1	(58)

Except for A2, all protein sequences were based on *L. donovani* sequences, as represented in GeneDB or GenBank. References to vaccine candidacy are not comprehensive, especially where multiple studies have been undertaken. Where possible we quote a reference where pre-clinical vaccine studies were undertaken in a model of visceral leishmaniasis.