HLA DR2b-binding peptides from human endogenous retrovirus envelope, Epstein-Barr virus and brain proteins in the context of molecular mimicry in multiple sclerosis

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Title: HLA DR2b-binding peptides from human endogenous retrovirus envelope, Epstein-Barr virus and brain proteins in the context of molecular mimicry in multiple sclerosis.

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Running Title: Molecular mimicry in multiple sclerosis

Abbreviations: ABP - α, β Crystallin; βSYN - β Synuclein; BLAST - Basic Local Alignment Search Tool; CNS – Central Nervous System; EAE- Experimental Autoimmune Encephalomyelitis; EBV – Epstein Barr Virus; EBNA1 - Epstein-Barr nuclear antigen 1; env – envelope; HERV – Human Endogenous Retrovirus; IEDB – Immune Epitope Data Base; MAG - Myelin-associated glycoprotein; MBP – Myelin Basic Protein; MOG - Myelin Oligodendrocyte Glycoprotein; MS – Multiple Sclerosis; MSRV - Multiple Sclerosis Associated Retrovirus; NCBI – National Center for Biotechnology Information; OSP – Oligodendrocyte Specific Protein; PLP - Proteolipid Protein; SMM - Stabilised Matrix Method; SYN1 - Syncytin-1; SYN2 - Syncytin-2; TCR – T cell receptor.

Abstract

Multiple sclerosis (MS) is a complex autoimmune disease in which T cells and antibodies damage the myelin sheath in the central nervous system. The aetiology of the disease is poorly understood. HLA Class II DR2b (DRB1*1501 β, DRA1*0101 α) is the strongest genetic risk factor for MS. Genetic remnants of ancient retroviruses, termed human endogenous retroviruses (HERV) that have been incorporated into the human genome and Epstein-Barr virus (EBV) infection have also been associated with MS. *In silico* analyses of human endogenous retroviral envelope (HERV env) proteins and three myelin proteins (myelin basic protein, myelin oligodendrocyte glycoprotein and proteolipid protein) that are principal targets of the autoimmune response showed homologies between potential T_H epitopes within pairs of viral and myelin peptides predicted to bind HLA DR2b. This led to the proposal that such molecular mimicry may potentially trigger MS. To further test this hypothesis, the HLA-DR2b binding characteristics of the three myelin proteins and HERV env peptides as well as *in silico* predicted peptides from other encephalitogenic brain proteins and EBV proteins were investigated. Peptides containing potential T_H epitopes from the myelin oligodendrocyte glycoprotein and HERV env previously predicted to bind HLA DR2b as well as other pertinent potential HLA DR2b-restricted epitopes were shown to be able to do so in a cell-free binding assay. Molecular modelling of HLA-DR2b in complex with high affinity peptides derived from MOG and HERV env proteins highlighted that prominent surface exposed amino acids, which potentially interface with the T cell receptor, are conserved. A structurally similar pair of potential T_H epitopes from the EBV protein EBNA1 and β synuclein, a brain protein implicated in MS, were shown to be similarly capable of binding HLA DR2b molecules. Our findings justify future investigation of T_H cell responses to the candidate peptides.

Key Words: autoimmunity; Epstein-Barr virus; HLA DR2b-peptide complex; human endogenous retroviruses; molecular mimicry; multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system (CNS) that involves progressive damage to the myelin sheath and axons leading to neurodegeneration [1 - 3]. Studies on MS patients and experimental allergic (autoimmune) encephalomyelitis (EAE) in rodents have implicated several CNS proteins present in oligodendrocytes and myelin, including the myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP), as targets of the autoimmune response in MS [1 - 3]. However, the aetiology of MS is not well understood. Cells of the innate immune system, $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells and autoantibodies are involved in the immunopathology of MS, while $CD4^+$ helper 1 cells (T_H1) among all types of antigen-specific cells, are considered to play the critical role in initiating the autoimmune process [1 - 3]. Environmental factors, *e.g.* vitamin D deficiency [4], and infections especially with Epstein Barr virus (EBV) [1-2, 5-8], have been implicated in predisposition to MS. Genome-wide association studies demonstrate that the HLA Class II allele DRB1*1501 β chain variant, which pairs with the relatively invariant DRA1*0101 α chain to form the HLA DR2b heterodimer in antigen-presenting cells (APCs), is the strongest genetic risk factor for MS [9]. The production of virions and expression of envelope protein (env) of a member of the genome-encoded human endogenous retrovirus W-family (HERV-W), termed the MS- associated retrovirus or MSRV [10], has also been implicated in MS [11 - 15]. However, the molecular mechanisms linking T_H cells to the genetic elements in the etiology of MS are not established. It has been recently hypothesized that epitopes in MSRV and other HERV family env proteins that cross-react with epitopes in myelin proteins, and presented by HLA DR2b on APCs to T_H1 cells in an inflammatory milieu, provide the requisite link [16].

Sequence homologies have been demonstrated by BLAST analysis between MBP, MOG and PLP on one hand and MSRV env on the other [16, 17]. In addition, structurally related sequences were found between the myelin proteins and syncytin-1

(SYN1) [16], another HERV-W family-derived env protein that has evolved to perform an essential role in forming the syncytiotrophoblast of the placenta [18]. SYN1 is 87% identical in amino acid sequence to MSRV env [16] and also more distantly related to syncytin-2 (SYN2), another essential fusogenic placental protein derived from a different HERV family termed HERV-FRD [19]. SYN2 also possesses regions of amino acid sequence similarity with the three myelin proteins [16]. SYN1 has an additional fusogenic role in the development of myotubes from myoblasts [20] and possibly osteoclasts [21].

In silico analyses of myelin and HERV env sequences utilizing the Immune Epitope Data Base (IEDB) [22] to predict HLA DR2b-binding 15mer peptides showed homologies between potential nonamer T_H epitopes within the 15mers from the HERV env proteins and all three myelin proteins that are predicted to bind to DR2b with high (IC_{50}<50nM) or intermediate affinity (50nM<IC_{50}<500nM) [16]. Homologies between a potential nonamer epitope in MOG and those in MSRV env, SYN1 and SYN2 were particularly significant [16]. Interestingly, some predicted higher affinity DR2b-binding peptides lie within longer regions of sequence homology between myelin proteins and HERV env proteins whilst others do not [16]. Since SYN1 and SYN2 have evolved to perform essential physiological functions in humans, it is possible that T_H cells that react with them may be deleted in the thymus and/or regulatory T cells (T_{regs}) that dampen an immune response are selected against them. This may not apply to MSRV env which is not expected to be normally expressed during development. However MSRV env is expressed within innate immune cells in an inflammatory situation *e.g.* during EBV infection [23] and is a potent stimulant of Toll-like receptor 4 present on macrophages and microglia, leading to impaired functional maturation of myelin-producing oligodendrocytes [24]. While existing data are consistent with an initiating role for molecular mimicry between the MSRV env and myelin proteins in MS, it is unclear whether this extends to the related SYN1 and SYN2 molecules. Once MS has been initiated in the proposed manner [16], further damage could

arise from T_H cells recognising other myelin epitopes presented by different HLA Class II molecules as a result of epitope spreading [16, 25].

This study experimentally investigated HLA DR2b binding of 15mer peptides derived from MBP, MOG, PLP and HERV env proteins earlier identified *in silico* as potentially able to bind to HLA DR2b [16]. It also examined the binding to HLA DR2b of selected peptides from additional CNS proteins reported to be encephalitogenic [1] and corresponding structurally related peptides present in the three HERV env proteins as well as EBV proteins that have been described to elicit prominent human $CD4^+$ T cell responses [2]. The binding of selected peptides with high HLA DR2b binding affinity were additionally examined by molecular modelling of peptide–HLA DR2b complexes.

2. Materials and Methods

2.1 Selection of CNS proteins for investigation

The three myelin proteins previously used for *in silico* analysis of peptides capable of binding to HLA DR2b [16] and six other potentially encephalitogenic myelin and oligodendrocyte-associated CNS proteins [1, 26] selected for the present study are listed in Table 1.

Table 1. CNS proteins selected for investigation

Protein	Abbreviation	NCBI sequence ID
Myelin basic protein	MBP	P02686.3
Myelin oligodendrocyte glycoprotein	MOG	Q16653.2
Phospholipid protein	PLP	P60201.2
α, β Crystallin	ABP	ACP18852
Myelin-associated oligodendrocyte basic protein	MOPB	NP_001265251.1
Oligodendrocyte-specific protein	OSP	AAC25187

2’3’ Cyclic nucleotide 3’ phosphodiesterase	CNPase	P09543
Myelin-associated glycoprotein	MAG	AAH53347.1
β-Synuclein	βSYN	Q16143

2.2 Selection of HERV and EBV proteins for investigation

The three HERV env proteins used previously for predicting HLA DR2b-restricted peptides through the IEDB *in silico* procedure [16] and EBV proteins reported to elicit strong human $CD4^+$ T cell responses [2] were selected for the present study (Table 2).

Table 2. Virus-derived proteins selected for investigation

Virus protein	**Abbreviation**	**NCBI sequence ID**
HERV-W Syncytin-1	SYN1	Q9UQF0
HERV-FRD Syncytin-2	SYN2	NP_997465
HERV-W Multiple sclerosis-associated retrovirus envelope protein	MSRV env	AAK18189.1
Epstein-Barr nuclear antigen 1	EBNA1	YP_401677.1
Epstein-Barr nuclear antigen 2	EBNA2	ALV83014.1
Epstein-Barr nuclear antigen 3C	EBNA3C	CEQ33769.1
Epstein-Barr virus transactivator BZLF1	BZLF1	CAD53423
Epstein-Barr virus glycoprotein BZLF2	BZLF2	CEQ33770.1
Epstein-Barr virus envelope glycoprotein H	BXLF2	ATE89094.1

2.3 Sequence homologies between CNS and virus-derived proteins

The predicted additional CNS and virus-derived protein coding sequences obtained from the US National Center for Biotechnology Information (NCBI) data base were compared by pairwise Basic Local Alignment Search Tool (BLASTp) analysis online using default parameters (https://www.ncbi.nlm.nih.gov/blast), as previously described for MBP,

MOG, PLP and the three HERV env proteins SYN1, SYN2 and MSRV env [16]. Additionally, every one of the selected CNS proteins shown in Table 1 were individually tested in BLASTp searches for homology against all non-redundant protein sequences of EBV (human herpes virus 4 strain B95-8) with NCBI taxonomy ID 10377.

2.4 Prediction of peptides potentially binding to HLA DR2b molecules

Prediction of potential peptides binding to HLA DR2b molecules was performed as previously described [13] using the IEDB analysis resource (www.iedb.org) [22, 27, 28]. The default peptide length of 15 amino acids was used in the analysis but the results also show the core nonamer peptides that are expected to bind to the HLA DR2b molecule and constitute the major portion of the T cell epitope [22, 27, 28]. The Stabilised Matrix Method (SMM) was used to rank the peptides according to their predicted binding affinities or IC_{50} which indicates the nM concentration of peptide expected to bind and achieve 50% saturation of the HLA DR2b molecules [22, 27, 28]. Structural similarities between core nonamer sequences in 15mer peptides from different proteins that were predicted to bind HLA DR2b with high or intermediate affinity (IC_{50}<50nM) were determined manually.

2.5 Determination of the binding affinity and stability of HLA DR2b-peptide complexes

Peptides (15mers) were synthesized by FMOC solid-phase chemistry and quality checked with matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) by ProImmune (Oxford, UK). Binding characteristics of the peptides to HLA DR2b were determined by ProImmune using the cell-free REVEAL® MHC class II binding assay [29]. The REVEAL® assay measured the ability of a peptide to stabilize the MHC-peptide complex based on the detection of the native conformation of the MHC-peptide

complex by a specific monoclonal antibody [29]. After an initial incubation with peptide for determining the proportion of MHC molecules binding the peptide (affinity), an additional measurement was taken after a further 24h incubation at 37^0C to determine the stability of binding (stability index). The stability index provides information on whether peptide can be presented long enough to serve as a T cell epitope. The affinity and stability index were measured as a percentage of the signal generated by the test peptide in comparison to a proprietary ProImmune positive control peptide. A well characterised 15mer MBP peptide with the sequence ENPVVHFFKNIVTPR (hereafter referred to as MBP_3) that is presented by HLA DR2b and activates $CD4^+$ T cells [30] was chosen as the internal comparative standard in the assays.

Details of the two sets of 40 peptides from CNS and viral proteins that were tested in the HLA DR2b binding assays are provided in Supplementary Table S1. The first set of 40 contained peptides derived from MBP, MOG and PLP and structurally similar peptides from SYN1 and MSRV env previously identified *in silico* as being potentially important for molecular mimicry by the IEDB algorithm [16]. Staggered arrays of 15mers were used to identify the best binding peptide. The first set also contained a 15mer derived from EBV DNA polymerase shown to cross-react at the $CD4^+$ T cell level with the control peptide MBP_3 on presentation by HLA DR2b [30]. A HLA DR2b-restricted MOG epitope shown previously to stimulate $CD4^+$ T cells to produce IFNγ [31] was also included in the first set of peptides. Other first set peptides were comprised of closely related signal sequence peptides of SYN1 and MSRV that encompassed structurally similar nonamers to those in internal peptides of MBP (including the control peptide MBP_3) and PLP [16], PLP peptides that contained the nonamer FFFLYGALL that were predicted to strongly bind DR2b [16], and four MSRV env peptides with the nonamer sequence TSVLVGPLV that exhibited weaker homology to MOG nonamer IVLPVLGPLV [16].

The second set of 40 peptides (Supplementary Table S1) were chosen to replicate and further examine the binding characteristics of the more promising HLA DR2b-

binding peptides identified from first set. They were independently synthesised and tested in REVEAL® binding assays. The second set additionally tested structurally similar pairs of HLA DR2b-binding peptides identified through IEDB *in silico* analysis in EBNA1 and HERV env proteins on one hand and different CNS proteins on the other. They included peptide pairs from EBNA1 and βSYN, as well as EBNA1 and OSP, that had also been independently predicted to bind HLA DR2b using a different *in silico* algorithm [32]. The second set also included a different MBP peptide reported to be recognised by T_H cells in the context of HLA DR2b [33].

2.6 Modelling of 15mer peptides binding to HLA DR2b

Molecular modelling of the HLA DR2b-peptide complexes were performed using the *in silico* docking program HADDOCK (high ambiguity driven protein-protein docking) [34]. Coordinates for the HLA DR2b complex were retrieved from the Protein Data Bank entry 1YMM [35]. Initial coordinates for the DR2b-restricted peptide moieties were extracted from the crystal structure of the T cell receptor(TCR)/HLA DR2b/MBP_3-peptide complex (entry 1YMM), and then used to build models of peptides with the molecular builder tool in COOT [36]. Each HLA DR2b-restricted peptide was subsequently subjected to a short regularisation protocol to ensure that the geometry of the peptide residues conformed to known bond lengths and angles.

The docking procedure was driven using only ambiguous intermolecular restraints, which were defined based on previously determined HLA DR2b-peptide complexes [35. 37, 38]. These structures revealed that the MBP_3 peptide is bound in the DR2b peptide-binding groove with peptide side chains P1, P4, P6 and P9 occupying pockets within the groove. Hence residues that line the P1, P4, P6 and P9 pockets of DR2b were selected as active residues (comprised of E11α, F24α, F32α, W43α, F54α, N62α, D66α, R76α, R13β, F26β, D28β, Q70β, A71β, Y78β, D57β and W61β). For the peptide only the

anchor residue side chains at P1, P4, P6 and P9 were defined as active residues. Passively involved residues were selected automatically. The 200 structures obtained after water refinement were analysed and ranked according to their HADDOCK score, a weighted sum of electrostatic, van der Waals, and restraint energy terms [34]. The lowest energy structure solutions were visualised and analysed using Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

3. Results

3.1 Sequence homologies between additional selected CNS and EBV or HERV env proteins

Sequence homologies between the three HERV env proteins and the three myelin proteins MBP, MOG and PLP observed in BLASTp analysis have been previously described [16]. BLASTp analysis of the non-redundant protein sequences coded in whole EBV genome against each of the selected CNS proteins only revealed a weak homology between α, β crystallin (ABP) and a 53 residue segment of the EBV protein EBNA4 with an E value of 0.95 (Supplementary Table S2). Pairwise BLASTp analysis of each of the other selected CNS proteins against the three HERV env proteins demonstrated homologies with E≤0.5 only between the pairs ABP and SYN2, ABP and MSRV env, and myelin-associated glycoprotein (MAG) and MSRV env (Supplementary Table S3).

3.2 Structurally related peptides in brain and EBV or HERV env proteins predicted to bind to HLA DR2b molecules

IEDB analysis of 15mer peptides containing structurally similar nonamers predicted to bind to HLA DR2b in MBP, MOG, PLP on one hand and the three HERV env proteins on the other, have been previously described [16]. Similar IEDB analysis performed

on the determined structurally similar regions of ABP/SYN2, ABP/MSRV env and MAG/MSRV env (Supplementary Tables S2 & S3) did not identify 15mer peptides of potentially high or intermediate affinity of binding to HLA DR2b that also contained structurally similar nonamer sequences in the three pairs of proteins (Supplementary Table S4 and reference 16).

Because of the homology observed between ABP and a 53 residue sequence of EBNA4 and perceived sequence similarities independently predicted between HLA DR2b-binding peptides of EBNA1 and several CNS proteins [32], HLA DR2b binding potential of 15mer peptides from EBNA1 and EBNA4 were also analysed by the IEDB procedure (Supplementary Table S5). These results when examined together with those in Supplementary Table S4 and data in reference 16 for HERV env, showed potential pairs of DR2b-binding peptides of high or intermediate affinity in EBNA1 and OSP, EBNA1 and βSYN, as well as OSP and MSRV env. These peptides whose sequences are given in Supplementary Table S6 were subsequently investigated in DR2b binding assays.

3.3 Experimental binding to HLA DR2b of CNS and viral peptides predicted *in silico* to bind HLA DR2b

The results of REVEAL binding assays on the selected peptides (Supplementary Table S6) showed that the pairs of peptides from MOG and the corresponding three HERV env proteins containing sequence-related nonamers previously predicted to engage HLA DR2b [16], and implicated in molecular mimicry, are able to bind HLA DR2b with comparable binding characteristics to the MBP_3 peptide.

The results also show that some OSP peptides with similar sequences to EBNA1 and MSRV env peptides and with predicted *in silico* intermediate binding affinity are able to bind well to HLA DR2b. However, the corresponding structurally related viral peptides did not reveal strong binding to HLA DR2b despite homology within the predicted nonamer

sequences. For example, the OSP 15mer STTLRALAPRLMRRV which bound strongly had five identities in its predicted nonamer DR2b-binding sequence (LRALAPRLM) to the corresponding nonamer (LRALLARSH) in two 15mer EBNA1 peptides that however only showed weak binding to DR2b (Supplementary Table S6).

Peptides from the closely related signal sequences of SYN1 and MSRV that contained sequence-related nonamers to those in internal peptides of MBP (including the control peptide MBP_3) and PLP identified in the previous study [16] did not bind strongly to DR2b in the assays. Only one PLP peptide TASFFFLYGALLLAE that contained the nonamer sequence FFFLYGALL that was predicted to bind strongly to DR2b [16] was confirmed to bind strongly to DR2b. Four MSRV env peptides tested containing the nonamer sequence TSVLVGPLV with weaker homology to the MOG nonamer IVLPVLGPLV did not bind strongly to DR2b.

Peptides from EBV DNA polymerase and a different MOG region that had been shown to be presented on DR2b and stimulate $CD4^+$ T cells [30, 31] revealed significant binding affinity to DR2b in the assay. A MBP peptide (GTLSKIFKLGGRDSR) containing a putative DR2b-resticted T cell epitope but with a weak predicted IC_{50} of 940nM based on IEDB analysis, only demonstrated marginal binding to DR2b.

An exact correlation between the *in silico* predicted affinity (IC_{50}) and the experimentally determined affinity by the REVEAL® binding assay for DR2b was not observed. For example, some PLP peptides with high predicted affinity (IC_{50}<1nM) showed poor experimental binding while four SYN2 peptides with predicted intermediate affinities (IC_{50} of 130 to 149nM) reveal experimental binding comparable to MBP_3 (Supplementary Table S6).

The details of binding assay results with staggered arrays of significant pairs of CNS and viral peptides are also shown graphically in Supplementary Table S6. Data on the

best binding 15mer peptides with nonamers relevant for molecular mimicry grouped together and compared with the binding of the control MBP peptide are listed in Table 3.

Table 3. Binding characteristics and Haddock scores of the best pairs of DR2b-binding 15mer peptides containing sequence-related nonamers relevant to molecular mimicry

Homology Group	Peptide	Peptide Sequence	Relative affinity	Relative stability	HADDOCK model score
1. MOG & HERV env	MOG_4	ITLFV**IVPVLGPLV**A	151	110	-123.7±2.5
	MSRV env _5	MPW**TLPFLGPLA**AII	69	33	-158.1±2.1
	SYN1_2	MPW**ILPFLGPLA**AII	144	124	-152.5±4.4
	SYN2_5	KWFSW**VLPLTGPLV**S	348	181	-137.8±5.3
2. βSYN & EBNA1	β synuclein	EKTKE**GVLYVGSKT**R	91	95	-128.7±3.0
	EBNA1_2	VAG**VFVYGGSKT**SLY	118	43	-131.5±5.1
3. Control	MBP_3	ENPV**VHFFKNIVT**PR	100	100	-163.7±1.6

Legend to Table 3. Results show the experimentally determined relative affinity and stability of binding of peptides expressed as a percentage of that observed with the control MBP_3 peptide assigned values of 100. The nonamers sequence predicted to bind in the peptide-binding groove in HLA DR2b are shown in bold letters and underlined. The docking scores for the HADDOCK-derived lowest energy HLA DR2b-peptide complex models are shown.

3.4 Molecular models of structurally similar peptides binding to HLA DR2b

Previously identified HLA DR2b-restricted peptides of similar sequences containing potential T_H epitopes from MOG and HERV env were shown to bind HLA DR2b in the cell-free binding assay. We employed *in silico* molecular docking strategies to understand the molecular mechanisms governing binding of the structurally related pairs of peptides by HLA DR2b and their potential recognition by TCR. To evaluate the feasibility of using such approaches we first modelled the binding of the control MBP_3 peptide

ENPVVHFFKNIVTPR to HLA DR2b using HADDOCK and then compared with the available crystallographic structure (PDB entry 1YMM) [35, 37, 38]. The HLA DR2b-MBP_3 complex model corresponding to the lowest intermolecular energy (with a HADDOCK score of -163.7) shows substantial similarity with the published structure in terms of epitope conformation and docking mode (Figure 1A). Superposition of the MBP_3 peptides derived from the published and model complex structures show that the main chain conformation is highly conserved (Figure 1A). In addition, similar to the published structure, the modelled MBP-3 peptide side chains at P1, P4, P6 and P9 serve as anchors slotting into the DR2b antigen binding cleft (Figure 1B&C). Finally, in both the published and modelled complexes, the peptide was held in the DR2 antigen-binding cleft by a conserved network of hydrogen bonding and non-polar interactions (Figure 1B&C). These observations justified the use of the HADDOCK docking approach to generate models of HLA DR2b bound to peptides that are relevant to MS.

To address the molecular mimicry hypothesis we generated models of HLA DR2b in complex with peptides of the highest affinity derived from MOG and the HERV env proteins MRSV env, SYN1 and SYN2 that are shown in Table 3. Superposition of the MOG, MSRV env, SYN1 and SYN2 peptides show that they all adopt a very similar back-bone conformation (Figure 2A). Similarly to the control MBP_3 peptide, the P1, P4, P6 and P9 peptide side chain positions serve as anchors inserting into the DR2b antigen binding cleft (Figure 2B-D). The HLA DR2b-peptide interactions were remarkably conserved between the different complexes including the control HLA DR2b-MBP_3 complex. In addition, positions P-1, P2, P5, and P8 are predicted to be surface exposed in the HADDOCK derived HLA DR2b-peptide complex models, and therefore potentially involved in binding to the TCR. The chemical characteristics of these prominent solvent exposed residues were either identical or structurally related in the relevant pairs of peptides. Taken together, these findings support the molecular mimicry hypothesis between MOG and HERV env proteins in triggering MS.

To further test the molecular mimicry hypothesis, *in silico* predicted peptides from other encephalitogenic brain proteins and EBV proteins were also investigated using modelling approaches. To address this HADDOCK derived models of HLA DR2b in complex with the βSYN and EBNA1 peptides shown in Table 3 were generated (Figure 3). These peptides adopted similar main chain conformations (Figure 3A) and mediated a conserved network of polar and non-polar interactions with side chains of DR2b (Figure 3 B&C). As with comparisons between MOG and HERV env proteins, the most prominent surface exposed residues (at P-1, P2, P5, and P8) and hence potential TCR contacts were mainly conserved or semi-conservatively substituted between the βSYN and EBNA1 peptide pair. The non-anchoring residues were however different between the two sets of unrelated peptide pairs βSYN/EBNA1 and HERV env/MOG, and between each of these and MBP_3 (Table 3 and Figures 1-3).

The HADDOCK docking scores of the best binding 15mer peptides possessing the relevant structurally related nonamer pairs are listed in Table 3. The HADDOCK scores do not correlate with experimentally measured REVEAL® binding affinities or stability indices for the peptides but the high negative values point towards energetically favourable binding to DR2b molecules.

4. Discussion

The molecular mimicry hypothesis proposed previously [16] attempted a unified explanation for the involvement of $CD4^+$ T cells, HLA-DR2b, HERV env proteins and EBV infection in the origin of MS. It was supported by the *in silico* identification of structurally related pairs of 15mer peptides predicted to bind DR2b in myelin-associated MBP, MOG and PLP proteins on one hand and HERV env proteins on the other. Homologies between predicted MOG and HERV env peptides were particularly prominent [16]. The present study extended the *in silico* predictions by examining the experimental binding of candidate 15mer

peptides to DR2b as well as generating molecular models of such complexes. It also investigated the presence of potential DR2b-binding, structurally related, peptide pairs between HERV env and other CNS proteins as well as between CNS and EBV proteins.

More recent findings are pertinent to the original HERV-related molecular mimicry hypothesis. EBV, which primarily infects B cells, has been further implicated as a necessary but not sufficient cause of MS, partly because of its increased and dysregulated expression in peripheral blood and brain [39 -43]. In addition, antibody titres to EBNA1 have lately been confirmed to be higher in MS patients compared to controls [44]. EBNA1 has recently been reported to promote alternative splicing of cellular genes [45]. Since EBNA1 is widely expressed in EBV infected cells [46], it is intriguing to speculate that its splicing activity has a role in the *trans* splicing that has been postulated to produce functional MSRV env molecules [17]. This adds to the many different mechanisms proposed to explain why EBV infections are a predisposition for MS [1-2, 5-8].

HERVs and their putative role in autoimmunity have been lately reviewed [47-49] and cross-reactive B cell epitopes in MOG and HERV-W env have been documented [50]. The presence of antibodies to HERV-W env proteins have recently been reported to differentiate MS from related neurological diseases [51, 52].

Evidence that human GDP-L-fucose synthase peptides are recognised by $CD4^+$ T cells in the context of HLA DRB3 *0202 in MS patients, and that gut bacterial GDP-L-fucose synthase may be cross-reactive has led to a different proposal for molecular mimicry in MS [53]. RAS guanyl releasing protein 2 in peripheral memory B cells driving the proliferation of brain-infiltrating $CD4^+$ T_H1 in a HLA DR2b-restricted manner that then recognise epitopes from the same protein expressed in brain cells has been proposed as another autoimmune mechanism explaining the association between MS and HLA DR2b [54].

Recent data also suggest that MSRV env is present in microglia associated with myelinated axons in MS lesions, MSRV env induces inflammatory myelin and neuron damaging activity in vitro in microglia and that antibodies to MSRV can be neuroprotective in MS patients [12]. These observations are pertinent to further examining molecular mimicry between MSRV env and MOG.

The present study suggests that peptides containing nonamers with potential T cell epitopes in MSRV env, SYN1, SYN2 and MOG have the capacity to bind to HLA DR2b molecules with comparable affinities and similar binding topology to the well characterised MBP_3 peptide containing a T cell epitope. The molecular modelling suggests that potential surface exposed residues that contact TCR are relatively conserved between the MOG and HERV env peptides which is consistent with the proposed molecular mimicry hypothesis. The MOG peptides identified that possessed DR2b-binding nonamers that were related in sequence to those in the three HERV env proteins are located in the predicted C terminal transmembrane domain of MOG. The corresponding DR2b-binding nonamers of related amino acid sequence from MSRV env, SYN1 and SYN2 are also sited in predicted transmembrane domains. A longer peptide from the transmembrane region of MOG, that contained the MOG peptide identified in the present work, has independently been shown to stimulate $CD4^+$ T cells from MS patients to proliferate and secrete IFNγ in a DRB-restricted manner [55]. It is possible that SYN1 and SYN2 may normally elicit tolerance as they may be recognised as self-proteins, while MSRV env may function as a foreign protein that can generate autoimmunity through molecular mimicry under certain circumstances as previously outlined [16]. Studies on CD4+ T cell response to the peptides identified in this study will help clarify the potential roles of MOG and the HERV env proteins in the immunopathogenesis of MS. It is relevant in this context that TCR recognition of MBP_3 bound to DR2b has been shown to involve skewed binding, not typical of TCR binding foreign peptide-Class II MHC complexes, which can result in potentially weaker interactions that may permit autoimmune T cells to escape deletion in the thymus [37].

412 This study did not find evidence for structurally related DR2b-resticted T cell
413 epitopes between HERV env proteins and other encephalitogenic CNS proteins. This was
414 also the case in our limited analysis of EBNA1 and EBNA4 against the selected CNS
415 proteins except for a pair of sequence-related nonamers derived from βSYN and EBNA1
416 that showed binding affinity and stability comparable to MBP_3 in the REVEAL® assay.
417 Modelling of the βSYN and EBNA1 peptides with HLA-DR2b revealed binding to the peptide
418 binding cleft similar to MBP_3 and relative conservation of the surface exposed, potential
419 TCR contact residues in the two peptides. This suggests the molecular mimicry is possible
420 between βSYN and EBNA1. It is relevant in this context that βSYN-reactive T_H cells have
421 recently been suggested to be responsible for autoimmune damage to CNS grey matter in
422 the progressive stage of MS [26]. The possibility that EBNA1 generated, βSYN-reactive T_H
423 cells induce additional autoimmune pathology, after the potential initiation of MS by
424 molecular mimicry between MOG and HERV env proteins, therefore justifies investigation.
425 Investigations on other pairs of potential HLA DR2b-binding peptides in EBNA1 and different
426 CNS proteins predicted independently [32] may also be useful in this context as the present
427 study was limited to CNS proteins with high encephalitogenic potential and restricted by the
428 numbers of peptide pairs that could be studied in the HLA DR2b binding assay.

429 The HLA DR2a molecule is formed by pairing of the DRB5*0101 β chain variant,
430 whose gene is closely linked to the DRB1*1501 gene in many individuals, with the relatively
431 non-polymorphic DRA1*0101 α chain. The previous *in silico* based predictions failed to
432 identify strong DR2a binding pairs of potential sequence-related T_H cell epitopes in HERV
433 env and myelin proteins MBP, MOG and PLP [16]. However because of the close genetic
434 linkage of the two β chain loci, the investigation of potential DR2a binding structurally similar
435 epitopes in the extended set of CNS proteins and EBV or HERV proteins is warranted
436 because DR2a and DR2b molecules bind complementary sets of peptides through different
437 binding motifs [56].

438

5. Conclusions

The results of the cell free HLA DR2b binding assays and molecular modelling show that sequence-related MOG and HERV env as well as βSYN and EBNA1 peptide pairs, with each set of pairs containing related potential T_H epitopes, are able to bind to HLA DR2b with similar affinity and conformation to a peptide MBP_3 containing an experimentally confirmed T_H epitope. These findings support the previous *in silico* analysis-based prediction that pairs of sequence-related peptides in HERV env proteins and MOG are potential candidates for a molecular mimicry origin of MS. Kinetic studies of HLA DR2b binding with highly purified peptides and determination of the crystal structure of HLA DR2b-peptide complexes can provide more comprehensive binding information in the future. However, definitive support for molecular mimicry will require detailed studies on $CD4^+$ T_H cell responses to the candidate peptides characterised in this study. Such investigations may also contribute to the variety of immunomodulatory approaches presently being explored for treating MS [12, 24, 57 - 63].

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

RR and UM initiated the project, FM performed the modelling studies, and RR did the IEDB analysis, collation of data and drafting of the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Figure 1 Comparison of the HLA DR2b-MBP_3 complex generated by HADDOCK with the reference structure. (A) Superposition of MBP_3 peptides bound to HLA DR2b in the reference (cyan) and modelled structures (black). (B) Ribbon representation of the published crystal structure of HLA DR2b bound to MBP peptide (MBP_3; ENPVVHFFKNIVTPR) (PDB entry 1YMM). (C) Ribbon representation of the lowest energy HLA DRb-MBP_3 complex model structure generated by HADDOCK. The HLA DR2b alpha and beta chains are depicted as pink and blue, respectively. For clarity only the peptide binding groove is highlighted. The peptide side chains (ball and stick format) and positions (red) are shown. Peptide residues P1, P4, P6 and P9 serve as anchor residues which slot into the antigen binding groove, whereas side

chains at P-1, P2, P5 and P8 are surface exposed. HLA-DR2b residues involved in stabilising peptide binding are also highlighted (ball and stick format). The black rectangle boxes correspond to the core 9-mer sequence for each peptide. Figure was generated with Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC)

Figure 2 Comparison of HADDOCK generated models of HLA-DR2b in complex with peptides derived from myelin (MOG) and HERV W-family (MSRVenv, SYN1 and SYN2) associated proteins. (A) Superposition of MOG_4 (red), MSRVenv_5 (blue), SYN1_2 (yellow) and SYN2_5 (green) peptides bound to HLA DR2b. (B) Ribbon representation of the lowest energy HLA DR2b-MOG_4 complex model structure. (C) Ribbon representation of the lowest energy HLA DRb-MSRV_5 complex model structure. (D) Ribbon representation of the lowest energy HLA DR2b-SYN1_2 complex model structure. (E) Ribbon representation of the lowest energy HLA DRb-SYN2_5 complex model structure. The HLA DR2b alpha and beta chains are depicted as pink and blue, respectively. For clarity only the peptide binding groove is highlighted. The peptide side chains (ball and stick format) and positions (red) are shown. Peptide residues P1, P4, P6 and P9 serve as anchor residues which insert into the antigen binding groove, whereas side chains at P-1, P2, P5 and P8 are surface exposed. HLA-DR2b residues that contribute to peptide interactions are also highlighted (ball and stick format). The black rectangle boxes correspond to the core 9-mer sequence for each peptide.

Figure 3 Comparison of HADDOCK generated models of HLA-DR2b in complex with peptides derived from a CNS (β-SYN) and an EBV (EBNA1) protein. (A) Superposition of βSYN (grey) and EBNA1_2 (orange) peptides bound to HLA DR2b. (B) Ribbon representation of the lowest energy HLA DR2b-βSYN complex model structure. (C) Ribbon representation of the lowest energy HLA DRb-EBNA1_2

complex model structure. The HLA DR2b alpha and beta chains are depicted as pink and blue, respectively. For clarity only the peptide binding groove is highlighted. The peptide side chains (ball and stick format) and positions (red) are shown. Peptide residues P1, P4, P6 and P9 serve as anchor residues which slot into the antigen binding groove, whereas side chains at P-1, P2, P5 and P8 are surface exposed. HLA-DR2b residues involved in peptide binding are also highlighted (ball and stick format). The black rectangle boxes correspond to the core 9-mer sequence for each peptide.