Role of a Novel Human Leukocyte Antigen-DQA1*01:02; DRB1*15:01 Mixed Isotype Heterodimer in the Pathogenesis of “Humanized” Multiple Sclerosis-like Disease*

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Background: HLA-DR15 haplotype (DRB1*15:01-DQA1*01:02-DQB1*06:02-DRB5*01:01) association with multiple sclerosis (MS) is conventionally attributed to effects from HLA-DRB1*15:01, with impact on MS risk from the neighboring HLA-DQ locus unclear.

Results: Functional studies show MS-like disease dependent on a novel DQA1*01:02;DRB1*15:01 mixed isotype heterodimer.

Conclusion: DQA1*01:02 within a mixed heterodimer may contribute to MS pathogenesis.

Significance: HLA class II/MS susceptibility models may require broader reinterpretation.

Gene-wide association and candidate gene studies indicate that the greatest effect on multiple sclerosis (MS) risk is driven by the HLA-DRB1*15:01 allele within the HLA-DR15 haplotype (HLA-DRB1*15:01-DQA1*01:02-DQB1*06:02-DRB5*01:01). Nevertheless, linkage disequilibrium makes it difficult to define, without functional studies, whether the functionally relevant effect derives from DRB1*15:01 only, from its neighboring DQA1*01:02-DQB1*06:02 or DRB5*01:01 genes of HLA-DR15 haplotype, or from their combinations or epistatic interactions. Here, we analyzed the impact of the different HLA-DR15 haplotype alleles on disease susceptibility in a new “humanized” model of MS induced in HLA-transgenic (Tg) mice by human oligodendrocyte-specific protein (OSP)/claudin-11 (hOSP), one of the bona fide potential primary target antigens in MS. We show that the hOSP-associated MS-like disease is dominated by the DRB1*15:01 allele not only as the DRA1*01:01;DRB1*15:01 isotropic heterodimer but also, unexpectedly, as a functional DQA1*01:02;DRB1*15:01 mixed isotype heterodimer. The contribution of HLA-DQA1/DRB1 mixed isotype heterodimer to OSP pathogenesis was revealed in (DRB1*1501xDQB1*0602)F1 double-Tg mice immunized with hOSP(142–161) peptide, where the encephalitogenic potential of prevalent DRB1*1501/hOSP(142–161)-reactive Th1/Th17 cells is hindered due to a single amino acid difference in the OSP(142–161) region between humans and mice; this impedes binding of DRB1*1501 to the mouse OSP(142–161) epitope in the mouse CNS while exposing functional binding of mouse OSP(142–161) to DQA1*01:02;DRB1*15:01 mixed isotype heterodimer. This study, which shows for the first time a functional HLA-DQA1/DRB1 mixed isotype heterodimer and its potential association with disease susceptibility, provides a rationale for a potential effect on MS risk from DQA1*01:02 through functional DQA1*01:02; DRB1*15:01 antigen presentation. Furthermore, it highlights a potential contribution to MS risk also from interisotypic combination between products of neighboring HLA-DR15 haplotype alleles, in this case the DQA1/DRB1 combination.

Multiple sclerosis (MS)2 is believed to result from pathogenic autoimmunity to several primary CNS target antigens (1). Among the different CNS components investigated, myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated oligodendrocytic basic protein (MOBP), and oligodendrocyte-specific protein (OSP)/claudin-11 (hereafter referred to as OSP) can be considered bona fide primary target antigens in MS in view of their demonstrated encephalitogenic potential in laboratory animals and the detection of autoimmune cells reactive against these antigens in MS patients (2).

1 The abbreviations used are: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; Tg, transgenic; OSP, oligodendrocyte-specific protein/claudin-11; hOSP, human OSP; mOSP, mouse OSP; phOSP and pmOSP, peptide of human mouse OSP, respectively; MBP, myelin basic protein; hMBP, human MBP; CFA, complete Freund’s adjuvant; PLP, proteolipid protein; MOG, myelin oligodendrocyte glycoprotein; MOBP, myelin-associated oligodendrocytic basic protein; MD, molecular dynamics; Ab, antibody; LN, lymph node; LNC, lymph node cell; APC, antigen-presenting cell; S.I., stimulation index.

* This work was supported in part by the Israel Science Foundation, National Multiple Sclerosis Society of New York Grant RG 319588/2, the Israel Ministry of Health, the Estate of the Late Florence Blau, the William Sahm Foundation, and the Sackler Faculty of Medicine, Tel-Aviv University, the Alzheimer Disease Association, the Alzheimer Society of Canada, and the Alzheimer Society of Alberta and the Northwest Territories. We thank Dr. C. Wicking for apraxia-sensitive variant MBP, and members of the Ben-Nun laboratory for helpful discussions. A.B.-N. is the Incumbent of the Eugene and Marcia Applebaum Professorial Chair. To whom correspondence should be addressed: Dept. of Immunology, Weizmann Institute of Science, 234 Herzl St., Rehovot 7610001, Israel. Tel.: 972-8-9344558; Fax: 972-8-9344141; E-mail: avi.ben-nun@weizmann.ac.il.

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MS is a disease with a strong underlying genetic component. Susceptibility has long been linked to the HLA-DR15 haplotype (DRB1*15:01-DQA1*01:02-DQB1*0602-DRB5*01:01) and, particularly, the HLA-DRB1*1501 locus, dominating MS risk in Caucasians (3, 4). Genome-wide association and candidate gene studies have suggested that the effects of the DR15 haplotype on MS risk are driven by the HLA-DRB1*15:01 allele and that effects on MS from neighboring alleles may reflect linkage disequilibrium (5, 6). However, linkage disequilibrium, particularly between DRB1*1501 and DQA1*0102-DQB1*0602, makes it difficult for genetic studies to discern, without functional studies, whether the functionally relevant effect on MS derives from DRB1*15:01 only, from its neighboring DQA1*01:02-DQB1*06:02 or DRB5*01:01 genes of the HLA-DR15 haplotype, or from their combinations or epistatic interactions. HLA-transgenic (Tg) mice constitute a valuable resource for dissecting the association of disease susceptibility with the specific gene products of the HLA-DR15 haplotype. The susceptibility of HLA-DRB1*1501-Tg mice to MBP- or MOG-induced EAE (7, 8) is consistent with the primary contribution of DRB1*1501 to MS risk. However, our recent studies showing that the DQB1*0602 allele, rather than DRB1*1501, determines disease susceptibility to MOBP (9) and to PLP (10), one of the most prominent and “MS-implicated” CNS target antigens, are inconsistent with the conclusion that the DR15 haplotype-associated MS risk is driven by the DRB1*15:01 allele only.

OSP/claudin-11, the third most abundant CNS myelin protein, comprising ~7% of total CNS myelin proteins (11, 12), is also a primary CNS myelin target antigen in MS (2), but, unlike MBP, PLP, MOG, and MOBP, neither the potentially pathogenic OSP epitopes relevant to HLA-DR15+ MS nor the HLA-DR15 haplotype gene product(s) that may determine OSP-related disease susceptibility has been investigated. In this study, HLA-DR15-Tg mice were employed first to determine immunogenic/immunodominant epitopes of human OSP/claudin-11 (hOSP) and their potential pathogenic relevance to HLA-DR15+ MS and, second, to study whether DRB1*1501 or DQA1*01:02-DQB1*06:02 alleles or their combinations control pathogenic T-cell autoimmunity against hOSP. We show that the DR15 haplotype-associated MS risk is driven by the DRB1*15:01 allele only.

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Experimental Procedures

Mice—HLA-DR15 (DRB1*15:01)-Tg mice (H-2Aβ−/) (referred to here as DRB1*1501-Tg) were generated by Dr. Cella David (14). The HLA-DQ6 (DQA1*01:02; DQB1*06:02)-Tg mice (H-2Aβ−/) (referred to here as DQB1*0602-Tg) were generated by Dr. Danny Altmann as described previously (15). Control HLA-II-Tg mice (HLA-DRB1*1502- and HLA-DQ6*0601-Tg) were generated by Dr. Cella David (16). The HLA-Tg mice used in this study are H-2Aβ−/− C57BL mice, which are homozygous for the HLA class II transgenes. Although capable of expressing H-2Aα and H-2β chains, HLA-Tg carrying the H-2Aβ−/− knockout may be considered phenotypically murine MHC class II null. All Tg lines were expanded at the animal facilities of the Weizmann Institute of Science under specific pathogen-free conditions, and progenies positive for the relevant transgenes by PCR were maintained. The expression level of HLA-DRB1*1501 and HLA-DQB1*0602 proteins by mononuclear splenocytes from DRB1*1501- and DQB1*0602-Tg mice, respectively, is shown in Fig. 10.

The HLA-DR15x1DQB1*0602)F1 double-Tg mice were bred at the animal facilities of the Weizmann Institute of Science. The Institutional Animal Care and Use Committee of the Weizmann Institute of Science approved the experiments, which were performed in accordance with its relevant guidelines and regulations.

Recombinant Human ΔOSP and OSP Synthetic Peptides—hΔOSP was constructed to delete the sequences encompassing the hydrophobic putative transmembrane domains (Fig. 1, a and b), thus enabling the expression of soluble protein. hΔOSP was produced by cloning the coding nucleotide sequences into pRSET bacterial expression vectors, as described previously for the preparation of mΔOSP (17), with the adjustment of some of the primers used for overlapping PCR extension to accommodate the few amino acid differences between mouse and human ΔOSP shown in Fig. 1 (in boldface type). Sequences were verified, and open reading frames for fusion protein with histidine tags were confirmed. Expression in Escherichia coli and purification of hΔOSP on Ni2⁺-nitriotretric acid-agarose was carried out as detailed in the study by Kerlero de Rosbo et al. (18). A recombinant protein named Y-DMP (Y-disease mellitus-related protein), a recombinant protein encompassing epitopes of several antigens relevant to diabetes3 that we expressed in E. coli and affinity-purified similar to the recombinant hOSP, was used as a non-relevant control recombinant protein. The amino acid sequences of synthetic overlapping peptides spanning hΔOSP are listed in Table 1.

Induction of EAE—Mice were injected subcutaneously at one site in the flank with 200 μl of emulsion containing 200 μg of hΔOSP or mΔOSP or 200 μg of peptide in complete Freund’s adjuvant (CFA) containing 300 μg of M. tuberculosis H37Ra (catalog no. 3114–25, Difco). Mice received 300 ng of pertussis toxin (catalog no. P-9452, Sigma) in 500 μl of PBS in the tail vein immediately and 48 h after the immunization (Protocol 1). In some cases, as indicated, the mice received an identical booster

3 A. Ben-Nun and N. Kerlero de Rosbo, unpublished data.
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immunization on the flank, 1 week later, but without the administration of pertussis toxin (Protocol 2). In another protocol, mice were injected intraperitoneally with cyclophosphamide (25 mg/kg) and 2 days later were inoculated in the flank with 200 μl of emulsion containing 200 μg of hΔOSP in CFA containing 300 μg of M. tuberculosis H37Ra (Protocol 3). Following the encephalitogenic challenge, mice were observed daily, and clinical manifestations of EAE were scored on a scale of 0–6 as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, flaccid tail; 3, hind leg paralysis; 4, hind leg paralysis with hind body paresis; 5, hind and fore leg paralysis; 6, death, as previously described (17).

Adaptive Transfer of EAE by T-cell Lines—Selection of T-cell lines and the cell transfer experiments were conducted as described previously (19, 20). Briefly, line T-cells were stimulated in vitro with their respective antigen for 3 days, and the activated T-cells were injected into the tail vein of irradiated (4 grays) naive syngeneic mice. Mice were observed and scored daily as described (17).

T-cell Proliferative Response—Mice were immunized with 150 μg of hΔOSP or individual peptides emulsified in CFA containing 150 μg of M. tuberculosis H37Ra (catalog no. 3114-25, Difco). 10 or 14 days postimmunization, draining lymph nodes or spleens, respectively, were removed and cultured in vitro in triplicates in microtiter plates, in the absence or presence of relevant antigens, as described previously (21). The cultures were incubated for 48–72 h at 37 °C in humidified air containing 7.5% CO2. [3H]Thymidine (1 mCi/well) was added for an additional 16 h of incubation, and the cultures were harvested and counted using a Matrix 96 Direct Beta Counter (Packard Instrument Co.). The results were expressed as stimulation index (S.I.) (mean cpm of antigen-containing cultures/mean cpm of medium-containing cultures). In some experiments, as indicated, mice were immunized as for induction of EAE (Protocol 1).

Cytokine Analysis—IL-2, IFN-γ, IL-4, and IL-10, were measured by ELISA according to standard protocols from PharMingen (San Diego, CA), as described previously (22). The capture antibodies were rat anti-mouse IL-2 (18191D, PharMingen), rat anti-mouse IL-4 (18161D, PharMingen), rat anti-mouse IL-10 (AMC0102, BioSource International, Camarillo, CA), and rat anti-mouse IFN-γ (AMC4834, BioSource International). The biotinylated antibodies used were rat anti-mouse IL-4 (18042D), rat anti-mouse IL-2 (18172D), rat anti-mouse IL-10 (18152D), and rat anti-mouse IFN-γ (18112D) (all from PharMingen). IL-17 was measured by ELISA using a DuoSet ELISA development kit (DY421, R&D Systems, Inc., Minneapolis, MN). TGF-β was measured by ELISA according to the standard protocol from R&D Systems, using recombinant human TGF-β RIIFc chimera as capture reagent (341-Br, R&D Systems) and biotinylated anti-human TGF-β1 antibody (BAF240, R&D Systems). Recombinant human TGF-β1 (240-B, R&D Systems) was used to construct the standard curve.

Affinity Binding Assays—Recombinant HLA-DRB1*1501 (DRA1*01:01;DRB1*15:01) and HLA-DQB1*0602 (DQA1*01:02;DQB1*06:02) proteins were expressed in Drosophila melanogaster cells (S2) under the control of the metallothionein promoter as soluble molecules engineered as described (23, 24). Proteins were antibody-affinity-purified from supernatants of HLA class II-transfected S2 cells. Briefly, supernatants were harvested from CuSO4-induced HLA class II-transfected S2 cells, centrifuged at 10,000 × g, and filtered through 0.4-μm filters before being passed over an anti-HLA-DR (L243) or anti-HLA-DQ (SPV-13) antibody-coupled column. After washing the column with 25 column volumes of PBS, 0.05% Nonidet P-40, captured HLA class II molecules were eluted with 4–5 column volumes of 0.15 M NaCl, 50 mM diethylamine, pH 11, directly into one-twenthieth volume of a neutralizing buffer (2 M Tris-HCl, pH 6.3). The eluate was then concentrated, and the buffer was changed to PBS, 0.1% NaN3 by ultracentrifugation in a Centrifcon p-20 unit (Millipore). The product was analyzed for purity by SDS-PAGE, and total protein concentration was determined by the bicinchoninic acid assay (Sigma) using bovine serum albumin (BSA) as a reference protein. Inhibition assays were performed essentially as described (24). Varying concentrations of competitor peptide were incubated overnight at 25 °C with constant concentrations of either HLA-DRB1*1501 (20 nm) plus biotinylated myelin basic protein peptide b-MBP (85–99) (ENPVVHFFKVTPR) (25) against peptide (10 nm) or HLA-DQB1*0602 protein (100 nm) plus biotinylated tyrosine phosphate b-IA2(495–509) (10 nm) in PBS containing 0.06% Nonidet P-40 and 0.1 M sodium citrate-phosphate buffer, pH 7.0. The formed DRB1*1501-peptide complexes or the HLA-DQB1*0602-peptide complexes were quantified by incubation for 2 h at 4 °C in a 96-well microtiter plate (MaxiSorb, Nunc, Roskilde, Denmark) precoated with 1 μg/well L243 (anti-DR) or SPV-L3 (anti-DQ) in carbonate buffer, pH 9.0, and blocked with 5% FCS in PBS. The free peptides and the unbound complexes were washed off of the plate with 0.05% Tween 20 in PBS. Europium-labeled streptavidin was added at 100 ng/ml and incubated for 1 h at 4 °C. After washing, Eu3+ was released by adding enhancement solution to the wells and measured in a time-resolved fluorometer (Vctor, PerkinElmer Life Sciences). IC50 values were monitored as the concentration of unlabeled peptide that prevented 50% of the biotinylated peptide from binding to the HLA-DRB1*1501 or the HLA-DQB1*0602 protein. For Fig. 6C, horseradish peroxidase-coupled streptavidin (Abcam) was diluted 5000-fold in washing buffer and added to the ELISA wells and incubated for 1 h at 4 °C. The substrate, 3,5,5′-tetramethylbenzidine (DAKO), was added and stopped after 15–30 min. with an equal volume of H2SO4, and the transformed substrate was measured at 450 nm.

Core Epitope Prediction and Molecular Modeling—Putative core epitopes within the hΔOSP and mΔOSP sequences were detected as described previously for I-A* molecules (18). Thus, the experimental structures of HLA-DRB1*1501(DRA1*01:01;DRB1*15:01) or -DQB1*0602 (DQA1*01:02;DQB1*06:02) molecules (Protein Data Bank codes 1BX2, 1YMM, and 2WB1 for DRB1*1501 and UVQ for DQB1*0602) were analyzed, and 4 × 20 binding preference matrices were constructed, which tabulate estimates of the tendency of an amino acid to bind in one of the specificity-determining pockets of the HLA binding site, P1, P4, P6, and P9. The binding estimates were based on the physical properties of the pockets, their size, hydrophobicity, polar-
ity, and charge. Thus, highly preferred residues were given a score of $-2$, less preferred residues were scored $-1$, residues that would neither contribute nor hinder the binding were scored 0, and residues that were likely to hinder binding weakly or strongly (e.g. by being too large) were given a score of 1 or 2, respectively. The binding preference matrices were used to estimate the binding ability of overlapping 9-amino acid segments of the target sequences to the HLA binding site. Previous experience with this in-house computer program successfully predicted core epitopes (18, 21, 22, 26), all of which scored $-5$ or lower. Higher scores were therefore disregarded in this study. An accurate estimate of the binding preferences in pocket P6 was obtained with ANCHORSMAP.2, a modified version of ANCHORSMAP (27), which also considers threonine and proline probes. This procedure accurately maps preferred binding positions and estimates the binding energies of excised amino acid side chains, taking into consideration that the amino acid is part of a protein. Anchoring spots with $\Delta G \leq -4$ kcal/mol were shown to correspond to particularly strong experimental hot spot locations (27), and values below $-3$ kcal/mol indicate good binding.

Initial model structures of the HLA peptide-binding domains in complex with peptides that are 12 amino acids long, which include a core epitope, were constructed based on the experimental structure of the DR15-peptide complex or DQ6-peptide complex. Experimental structures of hybrid HLA molecules are not available; therefore, models were constructed by superposing the experimental structures of DR15 and DQ6 using the corresponding Ca positions of the peptide binding domains and then combining the Ca chain of one HLA with the B chain of the other. Each initial model underwent a molecular dynamics (MD) simulation in water as follows: (i) initial energy minimization of the complex immersed in a box of water and neutralized; (ii) 200-ps MD simulation of the solvent molecules, restraining the complex. Experimental structures of hybrid HLA molecules are not available; therefore, models were constructed by superposing the experimental structures of DR15 and DQ6 using the corresponding Ca positions of the peptide binding domains and then combining the Ca chain of one HLA with the B chain of the other. Each initial model underwent a molecular dynamics (MD) simulation in water as follows: (i) initial energy minimization of the complex immersed in a box of water and neutralized; (ii) 200-ps MD simulation of the solvent molecules, restraining the non-hydrogen HLA and peptide atoms; (iii) 200-ps MD simulation of the solvent and peptide, restraining the non-hydrogen atoms of the HLA; and (iv) 1-ns MD simulation of the HLA, peptide, and solvent, restraining only the Ca atoms of the HLA. This procedure produced plausible models of the HLA-peptide complexes in which the HLA molecules mostly retained their initial structures, whereas the peptides could move. Convergence of the final MD simulation was tested by calculating the root mean square difference between steps along the simulation and the starting geometry, using all non-hydrogen atoms of the peptide after superposition of the HLA Ca atoms. We used the Gromac package for these computations (28). UCSF Chimera software (29) was used for visualization and figure preparation.

Pathological Examination—Mice were perfused with 4% paraformaldehyde in PBS, and the tissues were postfixed for 24 h at 4°C. Histological evaluation was performed on paraffin-embedded sections of brain and spinal cords that were sampled 19 days postimmunization as the experiment was terminated. Paraffin sections were stained with H&E and Luxol fast blue to assess inflammation and demyelination, respectively. In consecutive sections, immunohistochemistry was performed with Abs directed against the following targets: macrophages/activated microglia (MAC3, BD Pharmingen; iba-1, Wako-chem) and T-cells (CD3, Chemicon International) (18). For staining, paraffin sections were pretreated with a steamer for 60 min. Bound primary Ab was detected with a biotin-avidin technique as described in detail previously (18).

**Potential Effect on MS from DQA1*01:02; DRB1*15:01**

**Table 1**

| Peptides spanning hOSP<sup>a</sup> | hOSP<sup>a</sup> sequences | Amino acid sequences |
|---------------------------------|---------------------------|---------------------|
| OSP(22–46)                      | IVTTSTNEDMVTCGTYITPCKRKMDE|                      |
| OSP(35–55)                      | GYTPFTCPKGLDELSGKSLAD   |                      |
| OSP(45–66)                      | DELSGSKWACMVATGLYHCK    |                      |
| OSP(55–80)                      | DCMATGLYCHKVL1DILPGYQA  |                      |
| OSP(60–75)                      | POLHYCPEVL1L1LP         |                      |
| OSP(103–123)                    | LPCTBMQEPGVVTVLRQLA     |                      |
| OSP(142–161)                    | PFCVRHRTTVSGYSILA       |                      |
| OSP(179–201)                    | AGDAQAFRENFQAYTASSSSPPTH|                      |
| mOSP(142–161)                   | PFCVRHRTTVSGYSILA       |                      |

<sup>a</sup> All peptides were at least 80% pure. Peptides were synthesized in the laboratory of Prof. M. Fridkin (Weizmann Institute of Science), using the Fmoc (N-(9-fluorenylethoxycarbonyl) technique with an automated peptide synthesizer (AMS422, Abimed, Langenfeld, Germany).

**Results**

**HLA-DR15 Haplotype-related Immunogenic T-cell Epitopes of hOSP in HLA-Tg Mice**—To define HLA-DR15 haplotype-relevant immunogenic epitopes of human OSP, DRB1*1501- and DQB1*0602-Tg mice were immunized with each of the individual overlapping peptides (listed in Table 1) spanning the aqueous-soluble recombinant hOSP (Fig. 1; Δ, deleted of hydrophobic (transmembrane) domains). 10 days later, primed...
lymph node cells (LNCs) were analyzed ex vivo for a recall proliferative response to variable concentrations of the immunizing peptides. Fig. 2A shows that of the overlapping OSP peptides, only phOSP(142–161) elicited T-cell response in DRB1*1501-Tg mice, whereas immunized DQB1*0602-Tg mice showed T-cell reactivity only against phOSP(55–80) and phOSP(142–161) (Fig. 2B). These results suggest that h/J9004OSP harbors a single DRB1*1501-associated immunodominant epitope (or epitope cluster) encompassed within OSP(142–161) and two DQB1*0602-associated co-dominant epitopes (or epitope clusters) located within the OSP(55–80) and OSP(142–161) regions, which might be of relevance to autoimmunity against OSP in HLA-DR15+ individuals. Mapping the h/J9004OSP T-cell epitopes in non-HLA-DR15 haplotype-related control Tg mice showed that the DRB1*1502-Tg mice, which differ from DRB1*1501-Tg mice in only one amino acid residue (glycine for leucine substitution at position 86 (30)), significantly reacted to phOSP(142–161) and to phOSP(60–75), with the response to phOSP(142–161) being dominant (Fig. 2C). The non-HLA-DR15 haplotype-relevant HLA-DQ0601-Tg mice expressing the DQA1*01:03;DQB1*06:01 molecule reacted predominantly to co-dominant epitopes located within the OSP(45–66) and OSP(55–80) regions (Fig. 2D). These results suggest that the OSP(55–80) and OSP(142–161) regions of h/J9004OSP harbor the major HLA-DR15 haplotype-presented T-cell epitopes and their potential relevance to hOSP-associated pathogenic autoimmunity in HLA-DR15+/MS.

Analysis of the Binding Affinity of h/J9004OSP Overlapping Peptides to DRB1*1501 and DQB1*0602 Molecules—The finding that OSP(55–80) and OSP(142–161) regions encompass the epitopes of highest relevance to HLA-DR15-associated autoimmunity against hOSP was further studied in peptide binding competition assays using recombinant HLA-DRB1*1501 and HLA-DQB1*0602 molecules. As shown in Fig. 3, the DRB1*1501 molecule could bind several h/J9004OSP-derived peptides, of which phOSP(55–80), phOSP(103–123), and phOSP(142–161) showed the highest affinity (Fig. 3, A and C). The DQB1*0602 molecule bound only three of the h/J9004OSP-derived peptides, with moderate affinities. This includes phOSP(55–80), phOSP(142–161), and phOSP(179–201) (Fig. 3, B and C). These results suggest that phOSP(55–80),
phOSP(103–123), phOSP(142–161), and phOSP(179–201) encompass epitopes that can be potentially presented to CD4+ T-cells in the context of DRB1*1501 and/or DQB1*0602 on antigen-presenting cells (APCs) in vivo.

Determining the Immunodominant T-cell Epitopes of h/H9004 OSP in DRB1*1501 and DQB1*0602-Tg Mice—To validate the HLA-DR15 haplotype-relevant epitopes that had been defined by peptide immunization or HLA binding and to distinguish which epitopes were immunodominant and which cryptic, we studied DRB1*15:01- and DQB1*06:02-Tg mice immunized with recombinant h/H9004 OSP (Fig. 1). Fig. 4A shows that h/H9004 OSP was immunogenic in both DRB1*1501- and DQB1*0602-Tg mice, because the mice mounted a significantly higher specific T-cell response to h/H9004 OSP than to a control non-relevant recombinant protein, Y-DMP (a non-relevant recombinant protein encompassing epitopes of several antigens relevant to diabetes,3 which was expressed in E. coli and affinity-purified similarly to the recombinant hOSP). Fig. 4B shows that the T-cells reactive against the hOSP in DRB1*1501- and DQB1*0602-Tg mice were CD4+ and DRB1*1501- or DQB1*0602-restricted, respectively.

Ex vivo analysis of the recall proliferative responses of hΔOSP-primed spleen cells derived from hΔOSP-immunized DRB1*1501-Tg mice to the panel of overlapping peptides spanning the hOSP sequence consistently showed dominant T-cell reactivity against phOSP(142–161) only (Fig. 4C), similar to epitope mapping by peptide immunization (Fig. 2A). In contrast, the hΔOSP-primed spleen cells or LNCs from DQB1*0602-Tg mice showed no significant responses to any of the overlapping hΔOSP peptides (Fig. 4C), although these Tg mice could mount T-cell responses against phOSP(55–80) and phOSP(142–161) following peptide immunization (Fig. 2B), and the capacity of DQB1*06:02 to bind phOSP(55–80), phOSP(142–161), and phOSP(179–201) had been shown. The hΔOSP-immunized control non-HLA-DR15-related DRB1*1502-Tg mice and DQB1*0601-Tg mice showed immunodominant T-cell reactivity to phOSP(55–80) (Fig. 4C, bottom panels).

These results confirm that hΔOSP harbors a single DRB1*1501-associated immunodominant epitope encompassed by the OSP(142–161) region and suggest that the DQB1*0602-associated co-dominant epitopes encompassed by OSP(55–80) and OSP(142–161) regions of hΔOSP, which were identified by peptide immunization, are cryptic OSP epitopes for DQB1*0602-Tg mice.

Cytokine Profile of hOSP(55–80)- and hOSP(142–161)-reactive T-cells in DRB1*1501- and DQB1*0602-Tg Mice—To assess the encephalitogenic potential of the phOSP(142–161) region of DQA1*01:02;DRB1*15:01 and DRB1*15:01;DQB1*06:02 mice immunized with the relevant peptides (Fig. 1), we assessed their cytokine secretion profiles (Fig. 5). Our results showed that T-cells reactive against the hOSP in DRB1*1501- and DQB1*0602-Tg mice were CD4+ and DRB1*1501- or DQB1*0602-restricted, respectively.

In conclusion, the results presented in this study demonstrate that hΔOSP harbors a single DRB1*1501-associated immunodominant epitope encompassed by the OSP(142–161) region and suggest that the DQB1*0602-associated co-dominant epitopes encompassed by OSP(55–80) and OSP(142–161) regions of hΔOSP, which were identified by peptide immunization, are cryptic OSP epitopes for DQB1*0602-Tg mice.
and showed no clear pro- or anti-inflammatory characteristics (Fig. 5A). Analysis of phOSP(55–80)-reactive T-cells derived from DQB1*0602-Tg mice showed a tendency to anti-inflammatory characteristics, secreting IL-4 but not Th1/Th17 cytokines (IFN-γ or IL-17) (Fig. 5B).

The finding of Th1/Th17 pro-inflammatory reactivity against the immunodominant epitope within the OSP(142–161) region of hOSP in DRB1*1501-Tg mice, but not in DQB1*0602-Tg mice, and the propensity of DQB1*0602-Tg mice to develop Th2 anti-inflammatory T-cell reactivity against

| Peptide           | IC50 (nM)* DRB1*1501 | IC50 (nM)* DQB1*0602 |
|-------------------|----------------------|----------------------|
| hOSP22-46         | 1626                 | > 30000              |
| hOSP35-55         | 1192                 | 16300                |
| hOSP45-66         | > 30000              | > 30000              |
| hOSP55-80         | 93                   | 773                  |
| hOSP60-75         | 4571                 | > 30000              |
| hOSP103-123       | 75                   | > 30000              |
| hOSP142-161       | 29                   | 2800                 |
| hOS179-201        | 637                  | 390                  |
| MBBP84-102        | 41                   | nd                   |
| I-A2 (495-509)    | nd                   | 131                  |

*IC50 values represent the concentration of the competitor peptide leading to 50% inhibition.
the immunodominant epitope within the OSP(55–80) region of hOSP suggest that only the immunodominant epitope within the OSP(142–161) region may have encephalitogenic potential and only in DRB1*1501-Tg mice.

Analysis of the Encephalitogenic Potential of hOSP Immunodominant Epitopes in DRB1*1501- and DQB1*0602-Tg Mice—Epitope mapping and determination of the immunodominant epitopes of hOSP in DRB1*1501- and DQB1*0602-Tg mice, together with the analysis of the pro- and anti-inflammatory cytokine profile of the T-cell reactivity against the OSP(55–80) and OSP(142–161) immunodominant epitopes in these transgenics, suggested that only the immunodominant epitope within the OSP(142–161) region may have pathogenic potential in DRB1*1501-Tg mice. Nevertheless, we investigated the encephalitogenic potential of phOSP(142–161) as well as of phOSP(55–80) epitopes of hOSP in DRB1*1501-Tg- and DQB1*0602-Tg mice. However, we found that neither phOSP(142–161) nor phOSP(55–80) caused clinical EAE or
pathological manifestations in DRB1*1501-Tg- or in DQB1*0602-Tg mice (Table 2), despite the ability of DRB1*1501-Tg mice to mount Th1/Th17 T-cell reactivity against phOSP(142–161) (Fig. 5). Moreover, a highly reactive phOSP(142–161)-specific T-cell line that was derived from DRB1*1501-Tg mice, which showed a strong Th1/Th17 cytokine secretion profile (not shown), failed to transfer passive EAE to lightly irradiated (4 grays) naive syngeneic DRB1*1501-Tg recipients (Table 2).
Immunization with whole h\(\text{OSP}\) also failed to induce clinical or pathological EAE in DRB1*1501- or in DQB1*0602-Tg mice (Table 2).

In further attempts to demonstrate encephalitogenic potential of any of the immunogenic/immunodominant epitopes of h\(\text{OSP}\), non-HLA-DR15 haplotype-relevant controls (DRB1*1502-Tg and DQB1*0601-Tg mice) were also immunized to induce EAE by the h\(\text{OSP}\) peptides encompassing their relevant immunodominant epitope as well as by the whole h\(\text{OSP}\). As shown in Table 2, none of the transgenic mice expressing the HLA-DR15 haplotype-relevant alleles (DRB1*15:01 or DQB1*06:02) or the closely related control alleles (DRB1*15:02 or DQB1*06:01) were susceptible to EAE induced by their respective immunodominant epitope or by whole h\(\text{OSP}\).

The Encephalitogenic Potential of the Major Immunodominant Epitope of h\(\text{OSP}\) (142–161) in DRB1*1501-Tg Mice Is Hindered by Species Difference in the OSP Sequence—The results showing that the transgenic mice expressing the HLA-DR15 haplotype-relevant alleles (DRB1*15:01 or DQB1*06:02) are refractory to EAE induced by their respective immunodominant epitope or by whole h\(\text{OSP}\) (Table 2) may suggest that HLA-DR15-associated T-cell autoimmunity against OSP is not pathogenic. The DQB1*0602-Tg mice were expected to be refractory to EAE induction by the OSP(55–80) and OSP(142–161) immunodominant regions of h\(\text{OSP}\) not only because they represent cryptic epitopes in these Tg mice, but also due to their lack of pro-inflammatory T-cell cytokines against these immunodominant epitopes. In contrast, the consistent failure (Table 2) to induce EAE in DRB1*1501-Tg mice by phOSP(142–161) encompassing the sole immunodominant region of h\(\text{OSP}\) for this Tg mice remained enigmatic, particularly in view of the ability of DRB1*1501-Tg mice to generate potentially pathogenic Th1/Th17 cells reactive against phOSP(142–161) (Fig. 5).

Because pathogenic autoimmunity against the h\(\text{OSP}\) is analyzed in the HLA-Tg mice expressing CNS m\(\text{OSP}\), the failure in inducing EAE by phOSP(142–161) in DRB1*1501-Tg mice could be attributed to species differences between human and mouse OSP, although the hOSP(142–161) region (PVCAHREITIVSFGYSLYAG) differs from the mouse counterpart (PVCAHREITIVSFGYSVYAG) in only one amino acid residue (underlined). To investigate whether indeed this single residue difference between the human and mouse OSP(142–161) region underlies the inability of phOSP(142–161)-immunized DRB1*1501-Tg mice to develop EAE despite their ability to mount Th1/Th17 reactivity against the immunodominant hOSP(142–161) epitope, structural bioinformatics and functional studies were carried out. Using bioinformatics tools, overlapping 9-amino acid segments spanning h\(\Delta\text{OSP}\) sequence were analyzed for their binding preference to DRB1*1501 and DQB1*0602 (see “Experimental Procedures”). This scan detected a DRB1*1501-binding nonameric core epitope, residues 144–152, within the hOSP(142–161) region, and two DQB1*0602-binding nonameric core epitopes, one within the hOSP(55–80) region (residues 57–65) and another within the hOSP(142–161) region (residues 152–160). In contrast, a DRB1*1501-binding nonameric core epitope with preferred binding to DRB1*1501 was not detected within the mouse OSP(142–161) region (data not shown). The functional studies shown in Fig. 2 corroborated the computational analyses,

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**TABLE 2**

Study of the encephalitogenic potential of m\(\Delta\text{OSP}\) and h\(\Delta\text{OSP}\) peptides in HLA Tg mice

| HLA Tg mice | Peptide | Clinical incidence | Pathology |
|-------------|---------|--------------------|-----------|
| DRB1*1501   | h\(\Delta\text{OSP}\)^a | 0/3                | No pathological evidence |
|             | h\(\text{OSP}\)^a | 0/3                | ND^d      |
|             | m\(\Delta\text{OSP}\)^a | 0/3                | ND        |
|             | h\(\text{OSP}(142–161)^a\) | 0/4                | ND        |
|             | h\(\text{OSP}(142–161)^a\) | 0/6                | No pathological evidence |
|             | h\(\text{OSP}(142–161)^a\) | 0/3                | ND        |
|             | h\(\text{OSP}(142–161)^a\) line T cells (10 × 10^6) | 0/6                | ND        |

| DRB1*1502   | h\(\Delta\text{OSP}\)^a | 0/4                | ND        |
|             | h\(\Delta\text{OSP}\)^a | 0/6                | ND        |
|             | h\(\text{OSP}(55–80)^a\) | 0/3                | ND        |
|             | h\(\text{OSP}(60–75)^a\) | 0/4                | ND        |
|             | h\(\text{OSP}(142–161)^a\) | 0/4                | ND        |

| DQB1*0601   | h\(\Delta\text{OSP}\)^a | 0/3                | ND        |
|             | h\(\Delta\text{OSP}\)^a | 0/6                | ND        |
|             | m\(\Delta\text{OSP}\)^a | 0/3                | ND        |
|             | h\(\text{OSP}(55–80)^a\) | 0/4                | ND        |
|             | h\(\text{OSP}(45–66)^a\) | 0/4                | ND        |
|             | h\(\text{OSP}(142–161)^a\) | 0/4                | ND        |
|             | h\(\text{OSP}(55–80)^a\) | 0/3                | ND        |
|             | h\(\text{OSP}(179–201)^b\) | 0/7                | ND        |

| DQB1*0602   | h\(\Delta\text{OSP}\)^a | 0/3                | ND        |
|             | h\(\Delta\text{OSP}\)^a | 0/6                | ND        |
|             | m\(\Delta\text{OSP}\)^a | 0/6                | No pathological evidence |
|             | h\(\text{OSP}(55–80)^a\) | 0/4                | ND        |
|             | h\(\text{OSP}(142–161)^a\) | 0/4                | ND        |
|             | h\(\text{OSP}(60–75)^a\) | 0/4                | ND        |
|             | h\(\text{OSP}(142–161)^a\) line T cells (10 × 10^6) | 0/6                | ND        |

| (DRB1*1501xDQB1*0602)F1 | h\(\Delta\text{OSP}\)^a | 0/3                | ND        |

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^a Mice were injected with 200 \(\mu\)g of OSP peptide m\(\Delta\text{OSP}\) or h\(\Delta\text{OSP}\), and received 300 ng PT immediately and after 48 h.

^b Mice were injected with 200 \(\mu\)g of OSP peptide or h\(\Delta\text{OSP}\) and received 300 ng of PT immediately and after 48 h. Mice received a boost after a week.

^c Mice were injected intraperitoneally with cyclophosphamide (25 mg/l kg), and after 2 days mice received injection of 200 \(\mu\)g of h\(\Delta\text{OSP}\) in flank subcutaneously.

^d ND, not done.
predicted that the hOSP(142–161) region contains two different core epitopes, one for DRB1*1501 and another for DQB1*0602. (The latter epitope appeared to be cryptic (compare Fig. 4C with Fig. 2C).

Notably, the predicted DRB1*1501-binding nonameric core epitope 144–152 includes one of the very few residues that is not conserved between human and mouse OSP sequences, Thr-149 in human versus Ile-149 in mouse. To estimate the effect of the replacement of Thr → Ile in position 149 of hOSP on the recognition of mouse OSP(142–161) by DRB1*1501/hOSP(142–161)-reactive T-cells, we constructed models of the DRB1*1501 molecule in complex with the hOSP(144–152) peptide or with the corresponding mouse OSP(144–152) peptide (Fig. 6A), as described under “Experimental Procedures.”

**Figure 6.** Species difference in the OSP149 residue of hOSP(142–161) and mOSP(142–161) epitopes impair binding of mOSP(142–161) epitope to DRB1*15:01 heterodimer and its functional recognition by DRB1*15:01/hOSP(142–161)-reactive T-cells. A, a computational model (top (top panel) and side (bottom panel) views) of the peptide binding mode of hOSP(142–153) and mOSP(142–153) to DRB1*15:01, compared with hMBP(85–99)/DRB1*15:01 (experimental structure). The amino acid sequences of hOSP(142–161) and mOSP(142–161) epitopes are shown at the top of the figure (in boldface type is the predicted hOSP(144–152) nonameric core epitope, which differs from mOSP(144–152) only in the OSP149 residue (red). The semitransparent surface of DRB1*15:01 is colored by atom type: gray for carbon, red for oxygen, blue for nitrogen, and yellow for sulfur. Three peptides are shown: hMBP(85–99) (experimental) is shown with the carbon atoms in yellow; the carbon atoms in hOSP(144–152) peptide are colored in cyan; and in the mOSP(144–152) peptide, these atoms are colored in green. Nitrogen, oxygen, and sulfur atoms are colored in blue, red, and yellow, respectively. The side chains of residues that bind in pockets P1, P4, P6, and P9 are shown as ball-and-stick models. The side chains of residues p2 and p8 are omitted for clarity. The bottom panel presents a side view of the overlaid hMBP, hOSP, and mOSP peptides, showing the displacement of the C-terminal ends of the hMBP and the mOSP peptides out of pocket P9.

B, binding preferences in pocket P6 of DRB1*15:01. The ΔG of binding for Asn, Thr, and Ile in pocket P6 was calculated with ANCHORSMAP 2, as described under “Experimental Procedures,” showing that Ile is not a preferred residue for binding in this pocket and highlighting the difference between the human and mouse OSP epitopes. Note that Val-152 of hOSP (cyan) binds deep in pocket P9. C, the OSP149 difference between human and mouse OSP(142–161) region impairs the binding affinity of mOSP(142–161) to DRB1*15:01. The peptide binding competition assay was performed as described in Fig. 3A, except that the amount of formed DRB1*15:01 peptide complexes was monitored with horseradish peroxidase-labeled streptavidin and the substrate, 3,3',5,5'-tetramethylbenzidine. The enzymatic consumption of substrate was stopped with 1M H2SO4 and measured at 450 nm. The IC50 value was at least 10 times higher for mOSP(142–161) than that for hOSP(142–161) (in three binding competition assays).

D, hOSP(142–161) but not mOSP(142–161) stimulates HLA-DRB1*15:01-Tg-derived hOSP(142–161)-reactive T-cells. DRB1*15:01-Tg mice (2 mice/group) were immunized with 150 μg of hOSP(142–161) in CFA. Draining LNCs obtained 10 days later were analyzed ex vivo for their recall proliferative response to 1 or 2.5 μg/ml hOSP(142–161) or mOSP(142–161). The proliferative response was measured as described under “Experimental Procedures.” Results (S.I.) are the mean of two independent experiments with similar results obtained from pooled lymphocytes of two immunized HLA- Tg mice in each experiment.
where DRB1*1501 is bound to the MBP core epitope VHF-FKNIVT (Protein Data Bank codes 1BX2 and 1YMM) or to the microbial core epitope VHFISALHG (Protein Data Bank code 2WBJ), suggests a shift in the relative importance of the four major binding pockets in DRB1*1501, P1, P4, P6, and P9. P1 and P4 are dominant binding sites in the experimental structures, accommodating residues Val and Phe or Ile, respectively. In our model (Fig. 6A), these pockets accommodate OSP residues Cys-144 and Arg-147, respectively. The small side chain of OSP Cys-144 is adequate for the hydrophobic P1 pocket, but it makes fewer contacts than MBP Val-89. The hydrophobic part of the side chain of OSP Arg-147 interacts with the hydrophobic surface of pocket P4, and its positive end makes favorable contacts with the negative Asp 28β at the bottom of the pocket. Pocket P6 is polar, and in the experimental structures, it accommodates the MBP residue Asn-90 or Ala from the microbial peptide. Asn-90 interacts favorably with the hydrogen bonding network formed by DRB1*15:01 residues Arg-13β, Asp-28β, Tyr-30β, Glu-11α, and Gln-9α (31); however, its size and the binding directionality affect the conformation of the C-terminal part of the MBP peptide and hinder its binding. Thus, the hydrogen bonds between the side chain of DRB1*15:01 Asn-69α and the peptide backbone, common to all MHC class II-peptide complexes, are exceptionally long in this structure (>3.5 Å), and MBP residue Val-99 does not bind inside pocket P9. In the DRB1*1501-microbial peptide complex, P6 accommodates the small side chain of Ala, allowing formation of hydrogen bonds between DRB1*1501 Asn-69α and the peptide. However, Ala cannot participate in the hydrogen bonding network, and the Gly residue in position P9 of the viral peptide makes only minor contacts with pocket P9. In our model, pocket P6 accommodates residue hOSP Thr-149, which can interact with the hydrogen bonding network. Moreover, Thr is smaller than Asn, and its mode of binding allows formation of standard length hydrogen bonds between the peptide backbone and DRB1*1501 Asn-69α and binding of hOSP Val-152 inside pocket P9 (Fig. 6A). In contrast, the mouse OSP has a hydrophobic Ile residue in position 149, which cannot interact with the hydrogen bonding network in pocket 6 of DRB1*1501. Furthermore, Ile is larger than Thr, preventing formation of hydrogen bonds with DRB1*1501 Asn-69α and binding of Val-152 inside pocket P9. Computational mapping of anchoring spots (27) was used to determine the binding preferences of specific amino acids within the DRB1*1501 binding pockets (Table 3). The ANCHORMAP.2 results for Ile showed that it binds weakly in pocket P6, with ΔG of −2.7 kcal/mol compared with ΔG of −6.4 kcal/mol for Asn and −6.0 kcal/mol for Thr (Fig. 6B). In summary, whereas hMBP (85–99) binds strongly in pockets P1, P4, and P6, the human OSP (144–152) core epitope binds strongly to pockets P4, P6, and P9, and the corresponding mouse core epitope can bind strongly only to P4 (Fig. 6B). Thus, the mutation in position OSP149 (p6 of the OSP [144–152] core epitope) from Thr in humans to Ile in mice is likely to impede effective interaction with DRB1*1501.

Based on the modeling results, we concluded that the mouse OSP (142–161) peptide may bind poorly or not at all to DRB1*1501. This was confirmed by a peptide binding competition assay showing that mOSP (142–161) binds to DRB1*1501 with a lower affinity than the hOSP (142–161) (Fig. 6C). More importantly, this conclusion was also corroborated by functional analyses. hOSP (142–161)-primed LNCs derived from DRB1*1501-Tg mice immunized with phOSP (142–161) were analyzed ex vivo for their recall proliferative response to the human OSP (142–161) and to the mouse OSP (142–161) peptides. Fig. 6D shows that the DRB1*1501-derived, hOSP (142–161)-primed LNCs displayed a highly significant proliferative response only to phOSP (142–161) and not to the mouse OSP (142–161) peptide.

These results altogether resolve the lack of encephalitogenic activity by the DRB1*1501/OSP (142–161)-reactive Th1/Th17 cells in DRB1*1501-Tg mice and suggest that the hOSP (142–161) region constitutes the immunodominant epitope of OSP for HLA-DR15 haplotype-associated autoimmunity, the encephalitogenic potential of which is hindered in DRB1*1501-Tg mice by species-specific difference between human and mouse OSP amino acid sequence.

| HLA & peptide* | Anchoring spots energies§ |
|----------------|--------------------------|
|                | P1 | P4 | P6 | P9 |
| DRB1*1501      | exp VHF-FKNIVT            | V  -3.5 | F  -3.3 | N  -6.4 | T  -6.0 |
|                | hosp CAHRRTTV              | C  na  | R  -4.1 | T  -6.0 | I  -2.7 |
|                | mosp CAHRRTTV              | T  -5.0 | V  -3.6 |        |        |
| DRB1*1501      | exp VHFISALHG              | L  -4.0 | T  -3.8 | V  -5.0 | S  na  |
|                | hosp CAHRRTTV              | C  na  | R  -4.2 | T  -5.6 | I  -5.7 |
|                | mosp CAHRRTTV              |        |        |        |        |
| DRB15a/DQ6a    | exp LHFISALHG              | L  -4.0 | T  -4.4 | I  -2.7 |        |
|                | hosp CAHRRTTV              | C  na  | R  -3.6 | T  -4.0 | I  -3.9 |
|                | mosp CAHRRTTV              |        |        |        |        |
| DQA1/DQB1      | exp VHFISALHG              | C  na  | R  -3.6 | T  -4.0 | I  -3.9 |
|                | hosp CAHRRTTV              |        |        |        |        |
|                | mosp CAHRRTTV              |        |        |        |        |

* The underlined core epitopes are from the peptides bound to DRB1*1501 or DQB1*0602 in the experimental structures, Protein Data Bank entries 1BX2 and 1UVQ, respectively; a peptide from MBP is bound to DRB1*1501, and a peptide from hypoxerin is bound to DQB1*0602. The residues that bind in pockets P1, P4, P6, and P9 are shown in boldface type.

§ Cases of residues that cannot be accommodated within the designated pocket are indicated by "X" in the ΔG value column. ΔG for the small residues Cys, Ser, Ala, and Gly cannot be estimated with ANCHORMAP.2 and is indicated by "na". These small residues can readily bind in all four pockets but not as anchors.

ΔG for the result of the peptide binding experiments in the experimental structures show that the anchoring spots calculations identify correctly the locations of the observed anchors in P1, P4, P6, and P9 and their ΔG are low, as expected for strong binding.

| ANCHORMAP.2 results for Ile showed that it binds weakly in pocket P6, with ΔG of −2.7 kcal/mol compared with ΔG of −6.4 kcal/mol for Asn and −6.0 kcal/mol for Thr (Fig. 6B). In summary, whereas hMBP (85–99) binds strongly in pockets P1, P4, and P6, the human OSP (144–152) core epitope binds strongly to pockets P4, P6, and P9, and the corresponding mouse core epitope can bind strongly only to P4 (Fig. 6B). Thus, the mutation in position OSP149 (p6 of the OSP [144–152] core epitope) from Thr in humans to Ile in mice is likely to impede effective interaction with DRB1*1501.

Based on the modeling results, we concluded that the mouse OSP (142–161) peptide may bind poorly or not at all to DRB1*1501. This was confirmed by a peptide binding competition assay showing that mOSP (142–161) binds to DRB1*1501 with a lower affinity than the hOSP (142–161) (Fig. 6C). More importantly, this conclusion was also corroborated by functional analyses. hOSP (142–161)-primed LNCs derived from DRB1*1501-Tg mice immunized with phOSP (142–161) were analyzed ex vivo for their recall proliferative response to the human OSP (142–161) and to the mouse OSP (142–161) peptides. Fig. 6D shows that the DRB1*1501-derived, hOSP (142–161)-primed LNCs displayed a highly significant proliferative response only to phOSP (142–161) and not to the mouse OSP (142–161) peptide.

These results altogether resolve the lack of encephalitogenic activity by the DRB1*1501/OSP (142–161)-reactive Th1/Th17 cells in DRB1*1501-Tg mice and suggest that the hOSP (142–161) region constitutes the immunodominant epitope of OSP for HLA-DR15 haplotype-associated autoimmunity, the encephalitogenic potential of which is hindered in DRB1*1501-Tg mice by species-specific difference between human and mouse OSP amino acid sequence.
immunodominant epitope of hOSP also for these double-Tg F1 mice (Fig. 7A). Ex vivo analysis of the cytokine secretion profile of the phOSP(142–161)-reactive T-cells derived from phOSP(142–161)-immunized (DRB1*1501xDQB1*0602)F1 double-Tg mice suggested their pathogenic potential, as reflected by high secretion of pro-inflammatory cytokines (IL-17, INF-γ, and IL-2) and low secretion of anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) (Fig. 7B). Nonetheless, the phOSP(142–161)-immunized (DRB1*1501xDQB1*0602)F1 double-Tg mice were not expected to develop EAE because both parental Tg mice were refractory to phOSP(142–161)-induced EAE (Table 2). Moreover, phOSP(142–161) is cryptic for DQB1*0602-Tg mice, which anyway could not mount pro-inflammatory phOSP(142–161)-reactive T-cells (Fig. 5), and, more significantly, the potentially pathogenic DRB1*1501/phOSP(142–161)-reactive Th1/Th17 cells could not exert their pathogenic potential in parental DRB1*1501-Tg mice because the mutation to Ile149 in the mOSP(142–161) epitope impedes
their functional recognition of mOSP(142–161) (Fig. 6) in the CNS. Therefore, it was intriguing to find that (DRB1*1501xDQB1*0602)F1 double-Tg mice developed pathological EAE (Fig. 8 and Table 2) induced by phOSP(142–161). This raised the issue of how the double-Tg F1 mice developed CNS pathology and how the mouse OSP(142–161) epitope is recognized in the CNS by the potentially pathogenic hOSP(142–161)-reactive Th1/Th17 cells in the (DRB1*1501xDQB1*0602)F1 double-Tg mice, particularly because the major contribution to pathogenic activity in the double-Tg F1 mice could not come from the DQB1*0602/hOSP(142–161)-reactive T-cells or from the potentially pathogenic DRB1*1501/hOSP(142–161)-reactive Th1/Th17 cells, as detailed above.

Notably, however, the phOSP(142–161)-reactive T-cells derived from (DRB1*1501xDQB1*0602)F1 double-Tg mice, unlike phOSP(142–161)-reactive T-cells derived from parental DRB1*1501-Tg mice, responded to both the human and mouse OSP(142–161) epitopes to a similar extent (Fig. 7C). These unexpected findings, which were in contrast to the poor non-functional binding of mOSP(142–161) epitope to the DRB1*1501 molecule (Fig. 6), raised the possibility that phOSP-(142–161)-reactive T-cells cross-react with mOSP(142–161) through recognizing the mOSP(142–161) epitope in complex with a hybrid DR/DQ mixed isotype heterodimer, comprising the α chain of one HLA-II molecule and β chain of the other. phOSP(142–161)-reactive T-cells from (DRB1*1501xDQB1*0602)F1 Double-Tg mice Cross-react with mOSP(142–161) presented by DQA1*01:02;DRB1*15:01 Mixed Isotype Heterodimer—The possibility of cross-reactivity between phOSP(142–161) and pmOSP(142–161) in the context of their presentation through a hybrid DR/DQ mixed isotype heterodimer was first assessed by modeling the DRA1*01:01;DQB1*06:02 and DQA1*01:02;DRB1*15:01 (Fig. 9, A and B) hybrid heterodimers and analyzing their binding preferences to the mouse or human OSP(144–152) core epitope and thereby their potential presentation to T-cells. Modeling the binding of mouse and human OSP(144–152) to DRA1*01:01;DQB1*06:02 (Fig. 9A) predicted that this mixed isotype hybrid heterodimer can bind the human epitope but is less likely to bind the mouse epitope, because only Thr-149 of hOSP(144–152) and not Ile-149 of mOSP(144–152) is well accommodated in pocket P6 (G of −4.0 kcal/mol for Thr versus −2.7 kcal/mol for Ile; Fig. 9C and Table 3). In contrast, the mixed isotype heterodimer DQA1*01:02;DRB1*15:01 (Fig. 9B) is predicted to bind both mOSP(144–152) and hOSP(144–152) because Ile-149 and Thr-149 of mouse and human OSP(144–152), respectively, are accommodated equally well in P6 of the DQA1*01:02;DRB1*15:01 hybrid heterodimer (G of −4.0 and −3.9 kcal/mol for Thr and Ile, respectively, in P6 (Fig. 9C and Table 3). Hence, the computational analyses suggest that only the DQA1/DRB1 mixed isotype heterodimer can be associated with the disease induced in (DRB1*1501xDQB1*0602)F1 double-Tg mice by phOSP(142–161) because only DQA1*01:02;DRB1*15:01 can bind the CNS mouse OSP(144–152) but not the DRA1*01:01;DQB1*06:02 hybrid heterodimer (as depicted in Fig. 9, A–C, and Table 3) or the isotypic DRB1*15:01 heterodimer (Fig. 6).

Functional studies corroborated these predictions. Fig. 9D shows that phOSP(142–161)-reactive LN T-cells derived from phOSP(142–161)-immunized (DRB1*1501xDQB1*0602)F1 double-Tg mice react equally well to both mouse and human OSP(144–152).
Potential Effect on MS from DQA1*01:02;DRB1*15:01

FIGURE 9. mOSP(144–152) binds to the mixed isotype heterodimer DQA1*01:02;DRB1*15:01 but not to DRA1*01:01;DQB1*06:02. A and B, computational models (top view (top panel) and side view (bottom panel)) of the binding of hOSP(142–153) and mOSP(142–153) to DRA1*01:01;DQB1*06:02 (DR15/DQ6) and to DQA1*01:02;DRB1*15:01 (DQ6a/DR15) mixed isotype heterodimers. The coloring of the surface of the hybrid HLA and the peptides is as in Fig. 6. The side chain presentation is as in Fig. 6. Note that in DQ6a/DR15β, pocket 6 is mostly hydrophobic, accommodating both Ile-149 of mOSP and Thr-149 of hOSP. In contrast, in DR15α/DQ6β, the pocket is polar and allows better binding of Thr-149 than of Ile-149 (β and A side views, respectively). C, anchoring spot energies (ΔG in kcal/mol) of Thr and Ile side chains within pockets 6 of DR15α/DQ6β and DQ6a/DR15β mixed isotype dimers. ΔG values were calculated with ANCHORSMAP.2 (Table 3 and “Experimental Procedures”). The anchoring energies suggest similar binding capacity of hOSP and mOSP epitopes to DQ6α/DR15β but not to DR15α/DQ6β mixed isotype dimer. D, (DRB1*15:01xDQB1*06:02)F1 phOSP(142–161)-reactive T-cells cross-react with pmOSP(142–161) only in the presence of (DRB1*15:01xDQB1*06:02)F1 APCs. Non-adherent LN T-cells from HLA-(DRB1*15:01xDQB1*06:02)F1 double-Tg mice immunized with hOSP(142–161)/CFA were analyzed for their recall proliferative responses to the phOSP(142–161) and mOSP(142–161) in microtiter plate wells (in triplicates) in the presence of (DRB1*15:01xDQB1*06:02)F1 double-Tg mice.

Results (S.I.) are from pooled lymphocytes of two immunized HLA-Tg mice in one of two independent experiments showing similar results. E, (DRB1*15:01xDQB1*06:02)F1 phOSP(142–161)-reactive T-cells cross-react with pmOSP(142–161) in the context of DQ6α/DR15β in the absence or presence of blocking mAb specific for human HLA-DRα (L243), HLA-DRβ (Tal 14.1), HLA-DQα (L2), or HLA-DQβ (Genox3.5.3) or the respective isotype control Abs. % inhibition, (1 – (S.I. of proliferative response in the presence of neutralizing Abs/S.I. of the proliferative response in the absence of neutralizing Abs)) × 100. Data shown are the mean percentage inhibition values ± S.D. (error bars) from three independent experiments.

OSP(142–161) in the presence of irradiated (DRB1*1501xDQB1*0602)F1 APCs, whereas they were nonreactive to mOSP(142–161) in the presence of APCs from either DRB1*1501- or DQB1*0602-Tg mice. In contrast, in the presence of APCs from DRB1*1501, these F1-phOSP(142–161)-primed LN T-cells reacted only to phOSP(142–161) and not to mouse OSP(142–161) (in line with the results shown in Fig. 6D). These functional results strongly suggest that disease in the DRB1*1501xDQB1*0602)F1 double-Tg mice is associated with phOSP(142–161)-primed T-cells reactive against the mouse OSP(142–161) only when presented by the hybrid DR/DQ mixed isotype heterodimer, either DRA1*01:01; DQB1*06:02 or DQA1*01:02;DRB1*15:01 hybrid molecules.

To further determine experimentally which of these DR/DQ mixed isotype heterodimers is the restricting element for the response to mouse OSP(142–161), short term phOSP(142–161)-reactive line T-cells, derived from LN T-cells of phOSP(142–161)-immunized (DRB1*1501xDQB1*0602)F1 double-Tg mice, were analyzed for their proliferative response to mouse OSP(142–161) in the presence of (DRB1*1501xDQB1*0602)F1 APCs and in the absence or presence of monoclonal antibodies anti-DRα (L243), anti-DRβ (TAL14.1), anti-DQα (L2), and anti-DQβ (Genox3.5.3). Fig. 9E shows that only anti-DQα or anti-DRβ, and not anti-DRα or anti-DQβ, monoclonal antibodies effectively blocked the proliferative response to mouse OSP(142–161) by the (DRB1*1501xDQB1*0602)F1-derived, phOSP(142–161)-reactive line T-cells.

Collectively, these functional results, which are in line with the computational analysis (Fig. 9, A–C), strongly indicate functional expression of the hybrid DQA1*01:02;DRB1*15:01 mixed isotype heterodimer and its potential contribution to pathogenic OSP autoimmunity in the HLA-(DRB1*1501xDQB1*0602)F1 double-Tg mice.

Immunoprecipitation of DQA1*01:02;DRB1*15:01 Mixed Isotype Hybrid Heterodimer Expressed by Splenocytes of HLA-(DRB1*1501xDQB1*0602)F1 Double-Tg Mice—The studies detailed above indicate functional expression of the DQA1*01:02;DRB1*15:01 mixed isotype heterodimer and show its potential relevance to anti-myelin pathogenic autoimmunity in MS, albeit in HLA-(DRB1*1501xDQB1*0602)F1 double-Tg mice. Previous studies in different systems, however, could demonstrate the expression of only DRA1*01:01;DQβ1*06:02 and not...
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FIGURE 10. Expression of DQA1*01:02;DRB1*15:01 mixed isotype heterodimer by splenocytes of HLA-(DRB1*15:01xDQB1*06:02)F1 double-Tg mice. A, expression of DR and DQ molecules in parental and (DRB1*15:01xDQB1*06:02)F1 double-Tg mice. Nucleated splenocytes from the parental and double-Tg F1 mice were stained each with PE anti-human HLA DR (clone AB3, Dako 2014-10) and PE anti-human HLA-DQ (clone HLA-DQ1, Biolegend 318106). The percentage of DR- and DQ-expressing cells on gated mononucleated cells is shown. The FACS plots are from one representative experiment from three independent experiments. B, immunoprecipitation of hybrid DQA1*01:02;DRB1*15:01 mixed isotype heterodimers. Nucleated splenocytes derived from parental and F1 double-Tg mice were extracted, and the whole cell lysate was immunoblotted (IB) with anti-HLA-DQA1 (EPR7300, Abcam) or anti-GAPDH antibody as a control (WCL, left blot) or was subjected first to immunoprecipitation (IP) with anti-HLA-DRβ antibodies (TAL 14.1, Santa Cruz Biotechnology).

Discussion

MS is a disease with a strong underlying genetic component and in which CNS demyelination presents as heterogeneous clinical and pathological phenotypes. The basis for the divergent patterns of clinical manifestation is unclear, but it may be a reflection of variable modes of CNS tissue damage associated with different pathways of pathogenic anti-CNS autoimmunity directed against diverse CNS myelin components (1). In view of the multiplicity of potential primary CNS target antigens in MS (including MBP, PLP, MOG, MOBP, and OSP) (2), a different major CNS target antigen/epitope against which pathogenic autoimmunity is primarily directed in different patients and/or different patients’ HLA genotype could be a major factor underlying the heterogeneous disease phenotype. This highlights the value of characterizing the disease phenotype correlates of patterns of peptide/HLA class II antigenic presentation. This, however, requires definition of MS patient target antigens and major pathogenic epitope in the context of patients’ HLA genotypes. Such characterization would be of significance also for devising immune effector-specific therapeutics for MS as well as for investigating disease etiology, in view of the possibility that cross-reactive immunity between viral/bacterial components and mimotopes of CNS antigen(s) may lead to MS in individuals with permissive genotypes (1, 34, 35).

Studies in HLA-DR15-Tg mice have identified potentially pathogenic epitopes of MBP, PLP, MOG, and MOBP, relevant to HLA-DR15+ MS, and indicated the heterodimers from the DR15 haplotype that may potentially govern pathogenic autoimmunity against these primary target antigens in HLA-DR15+ MS (7–10). OSP/Claudin-11 is also a bona fide potential primary target antigen in MS in view of its encephalitogenic potential in SJL/J, C57Bl/6J, and ABH mice (17, 22, 26, 36, 37) or rhesus monkeys (38) and the detection of anti-OSP antibodies and OSP-reactive T-cells in MS patients (39, 40). The studies detailed above identified the hOSP(142–161) epitope as the single immunodominant epitope of hOSP and suggested its potential relevance to pathogenesis in HLA-DR15+ MS, with the DRB1*15:01 gene product dominating the pathogenic autoimmunity against OSP, although DRB1*1501/hOSP(142–161)-reactive Th1/Th17 cells were not pathogenic in DRB1*1501-Tg mice. However, structural bioinformatics analyses, supported by experimental data, showed that their encephalitogenic potential is hindered by a single, but critical, amino acid difference in OSP(142–161) regions of human and mouse OSP, Thr versus Ile at position 149. In silico and experimental analyses of the binding mode to DRB1*1501 showed that the mouse Ile-149, unlike human Thr-149, interferes with binding of the mouse OSP(144–152) nonameric core epitope to the DRB1*1501 isotypic heterodimer and thereby impedes the functional recognition of the mOSP(142–161) epitope in the CNS of DRB1*1501-Tg mice by potentially pathogenic DRB1*1501/hOSP(142–161)-reactive Th1/Th17 cells. Therefore, the path-
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The genetic potential of these hOSP(142–161)-reactive T-cell cannot be ignored, and, as detailed above, the DRB1*15:01 allele may contribute to OSP-associated pathogenic autoimmunity both as DRB1*15:01 isotypic heterodimer and as a DQA1*01:02;DRB1*15:01 mixed isotype heterodimer.

The strong association of HLA-DR15 haplotype with susceptibility to MS has long been recognized. Genome-wide association and candidate gene studies have suggested that the greatest effects on MS risk conferred by the DR15 haplotype are driven by the HLA-DRB1*15:01 allele (5, 6, 41, 42). However, linkage disequilibrium in the HLA-class II region makes it difficult without functional studies to distinguish whether the functionally relevant effect on MS derives only from the DRB1*15:01 or also from the neighboring genes in the HLA-DR15 region, from their combination, or from their epistatic interaction. Many genetic or immune functional studies implicate DRB1*15:01 as the primary risk factor in MS (5, 41, 43) while suggesting only a disease-modifying role for the DRB5*01:01 or DQB1*06:02 alleles (44, 45). In this respect, the studies presented here showing the dominant role of DRB1*15:01 in OSP autoimmunity in HLA-Tg mice, together with previously reported susceptibility of DRB1*1501-Tg mice to MBP- or MOG-induced EAE (7, 8), are consistent with a primary contribution of the DRB1*15:01 allele to MS risk in HLA-DR15+ individuals. However, our recent findings that the DQB1*06:02, rather than the DRB1*15:01 allele, determines susceptibility to EAE induced by MOBP (9) or PLP (10) offer a rationale for a disease-predisposing role also for DQB1*06:02. Thus, the reductionist transgenic models of MS suggest that for HLA-DR15+ MS, the disease susceptibility associated with pathogenic autoimmunity against MBP, PLP, and OSP is determined by DRB1*15:01, whereas that against MOBP or against the PLP (one of the most prominent “MS-implicated” CNS target antigens) is dominated by DQA1*01:02;DQB1*06:02 alleles of the HLA-DR15 haplotype. These data suggest a more complex and differential functional role for HLA class II isotypes from the DR15 haplotype, depending on the primary target myelin antigen under attack. The finding that DRB1*15:01 may determine OSP-associated pathogenic autoimmunity not only as the classical DRA/DRB1 heterodimer, but also as a DQA1*01:02;DRB1*15:01 mixed isotype heterodimer, introduces further complexity to the potential contribution of the DR15 haplotype alleles to MS risk. Although confined to transgenic models, these findings altogether strongly support the possibility that the functionally relevant genetic impact on MS can be driven by effects from the DRB1*15:01 locus, from its neighboring DQB1*06:02, and also from an interisotypic combination between alleles of DR and DQ loci, depending on the primary target myelin antigen against which the pathogenic autoimmunity is primarily directed.

The involvement of the DQA1*01:02;DRB1*15:01 mixed isotype heterodimer in OSP autoimmunity could not be revealed in hOSP(142–161)-immunized (DRB1*1501xDQB1*0602)F1 double-Tg mice that developed EAE-like CNS pathology if there were no species differences between hOSP(142–161) and mOSP(142–161). EAE-like disease developed in these double-Tg mice, despite the hindrance of the encephalitogenic potential of the predominant DRB1*1501/hOSP(142–161)-reactive Th1/Th17 cells due to a single amino acid difference between human and mouse OSP(142–161) region, which impedes binding of DRB1*1501 to the mOSP(142–161) epitope in the CNS of HLA-Tg mice. The unexpected EAE pathology in the F1 double-Tg mice raised the intriguing possibility that phOSP(142–161)-immunized (DRB1*1501xDQB1*0602)F1 double-Tg mice could generate a phOSP(142–161)-reactive T-cell subpopulation that cross-reacts with mOSP(142–161) presented by a hybrid DR/DQ mixed isotype heterodimer. In support of this, phOSP(142–161)-reactive T-cells from F1 double-Tg mice cross-react with mOSP(142–161) only in the presence of (DRB1*1501xDQB1*0602)F1 APCs and not in the presence of either parental APCs. In silico modeling of DRA1*01:01xDQB1*06:02 and DQA1*01:02;DRB1*15:01 hybrid heterodimers and analysis of their binding preferences to the mouse or human OSP(144–152) core epitope suggested that mixed isotype hybrid DRA1/DQB1 is likely to bind human OSP(144–152) but not the mouse OSP(144–152) core epitope, whereas the hybrid DQA1/DRB1 can bind both mouse and human OSP(144–152).

There has previously been consideration of mixed heterodimers in murine EAE. In MBP-immunized (PL/xSIL/J)F1 mice, MBP-reactive T-cells restricted to hybrid AαAββ or to EaαEββ heterodimers were identified, in addition to MBP-reactive T-cells restricted by EaαAββ and EaαEββ dimers (46). Also, isotype-mismatched EaαAββ or EaαEββ hybrid MHC-class II heterodimers that were functional in stimulating antigen-reactive T-cells have been identified (47–49), but none of these studies reported Aα-Eβ (the DQA-DRB human orthologs) mixed isotype heterodimers. Human HLA class II isotype mixed pairing has also been previously reported (32, 33, 50, 51). Lotteau et al. (32, 33) identified the existence of mixed isotype DRα-DQβ hybrid dimers in B-cell lines but could not identify any DQα-DRβ mixed isotype dimers. Recently, mixed DQα-DRβ isotype hybrid molecules (BoLA-DQA/DRB3) that were functional in presenting viral peptide to CD4+ T-cells have been reported in cattle immunized with Anaplasma marginale vaccine (52).

Our in silico analysis suggesting that the DQA1*01:02;DRB1*15:01, and not DRA1*01:01xDQB1*06:02, mixed isotype heterodimer may be implicated in OSP pathogenesis in (DRB1*1501xDQB1*0602)F1 double-Tg mice was confirmed by functional studies. The findings that the cross-reactive response to mouse OSP(142–161) by phOSP142-reactive T-cells derived from (DRB1*1501xDQB1*0602)F1 double-Tg mice is blocked by anti-DQα or anti-DRβ, and not by anti-DRα or anti-DQβ, blocking monoclonal antibodies (Fig. 9) firmly supports the functional recognition of mouse OSP(142–161) presented by DQA1*01:02;DRB1*15:01 mixed isotype heterodimer. The immunoprecipitation and Western blot analyses (Fig. 10) confirmed the stable expression of the DQA1*01:02;DRB1*15:01 mixed isotype heterodimer by (DRB1*1501xDQB1*0602)F1 double-Tg mice.

The existence of isotype-matched and isotype-mismatched hybrid HLA-DR/DQ heterodimers may considerably increase the repertoire of HLA antigens associated with susceptibility to autoimmune diseases. The case of OSP-associated pathogenic autoimmunity in (DRB1*1501xDQB1*0602)F1 double-Tg mice
strengthens this possibility because both DRB1*15:01 isotypic heterodimer and DQA1*01:02;DRB1*15:01 mixed isotype heterodimer were associated with disease susceptibility. The DQA1*01:02, but not the DQB1*06:02, allele is prevalent also in individuals with non-HLA-DR15 haplotypes (13). In a large family-based investigation of HLA-II loci in MS, it was found that DQA1*01:02 increased disease risk through epistatic interaction with DRB1*15:01 (13).

Our demonstration of functional interaction between DQA1*01:02 and DRB1*15:01 in the humanized model of MS provides a rationale for a mechanism by which the DQA1*01:02 allele may contribute to MS risk also as a DQA1*01:02;DRB1*15:01 mixed isotype heterodimer functional in antigen presentation. However, although these observations highlight the principle, further studies will be needed to establish the extent to which this novel mixed isotype heterodimer contributes to the autoimmune repertoire and particularly the contribution of self-OSP epitope presentation by mixed isotype or by isotypic heterodimers to the autoimmune repertoire of human MS patients.

Overall, our transgenic model suggests that the potentially pathogenic autoimmune immunity against OSP in HLA-DR15+ MS is likely to be focused on a single immunodominant epitope, OSP(142–161) (144–152 core epitope), that is governed by the DRB1*15:01 allele, not only as the DRB1*15:01 isotypic heterodimer but also as a functional DQA1*01:02;DRB1*15:01 mixed isotype heterodimer. These studies, which demonstrate for the first time, to the best of our knowledge, a functional HLA-DQA1/DRB1 mixed isotype heterodimer, provide a rationale for the effect of DQA1*01:02 on MS through QA1*01:02;DRB1*15:01 antigen presentation and highlight a potential contribution to MS also from interisotypic combination between HLA-class II monomers of DQ and DR alleles of the HLA-DR15 haplotype.

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