HLA-DQB1*0602 Determines Disease Susceptibility in a New “Humanized” Multiple Sclerosis Model in HLA-DR15 (DRB1*1501;DQB1*0602) Transgenic Mice

Nathali Kaushansky,* Daniel M. Altmann,† Stephanie Ascough,‡ Chella S. David,§ Hans Lassmann,** and Avraham Ben-Nun†*†

The susceptibility to multiple sclerosis (MS), a chronic neurological autoimmune disease that primarily targets CNS myelin, has long been associated with HLA class-II genes. Although several other HLA and non-HLA disease predisposing alleles have been identified, alleles of the HLA-DR15 haplotype (DRB1*1501, DRB5*0101, and DQB1*0602) remain the strongest susceptibility factor. Many studies have suggested that the HLA-DRB1*1501 allele determines MS-associated susceptibility. However, due to strong linkage disequilibrium within the HLA class II region, it has been difficult to unequivocally determine the relative roles of the DRB1*1501 and DQB1*0602 products. In this study we use HLA class-II transgenic mice to illuminate the relative contributions of the DRB1*1501 and DQB1*0602 alleles or their combination to susceptibility toward a new “humanized” MS-like disease induced by myelin-associated oligodendrocytic basic protein (MOBP). Although many immunological studies have focused overwhelmingly on the role of the HLA-DRB1*1501 product in MS, we show that HLA-DRB1*1501 transgenics are refractory to MOBP disease induction, whereas the HLA-DQB1*0602 transgenics are susceptible via T cells reactive against MOBP15–36 and MOBP55–77 encephalitogenic epitopes. Although both transgenics react against these epitopes, the MOBP15–36- and MOBP55–77-reactive T cells are of Th2-type in HLA-DRB1*1501 transgenics and are pathogenic Th1/Th17 cells in the HLA-DQB1*0602 transgenic mice. This new humanized model of MS further implicates autoimmunity against MOBP in MS pathogenesis, provides the first evidence of pathogenic HLA-DQ-associated anti-myelin autoimmunity, and is the first to offer a rationale for HLA-DQB1*0602 association with MS. These findings have important bearing on the candidacy of the DQB1*0602 allele as a genetic risk factor for MS. The Journal of Immunology, 2009, 183: 3531–3541.

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS associated with demyelination, astrogliosis, and varying degrees of axonal damage and neuronal loss. Although the etiology of the disease is still uncertain, ample evidence suggests that autoimmunity against CNS components plays a pathogenic role in MS (2), and it is widely accepted that abnormally activated autoimmune T cells recognizing components of CNS myelin may cause the initiation and progression of MS (1–3). Among the different CNS myelin components that have been investigated as potential target Ags in MS, myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and, more recently, also myelin-associated oligodendrocytic basic protein (MOBP) and oligodendrocyte-specific protein (OSP)/claudin-11 can be considered, thus far, as bona fide primary target Ags in MS in view of their ability to induce overt clinical experimental autoimmune encephalomyelitis (EAE) in laboratory animals and the detection of autoimmune cells reactive against these proteins in MS patients (4).

Numerous genetic and epidemiological studies show that both genetic and environmental factors play a role in the etiology of MS (5, 6). For >30 years it has been clear, first from serological HLA typing and then from DNA genotyping, that there was an association with the HLA class II region. Despite intensive efforts aimed at identifying genetic or environmental factors associated with susceptibility to MS, the strongest MS risk factors remain those within the MHC class II (MHCII) region. During the past decade there have been exhaustive genome scans of DNA from MS patients. Although there is a contribution to MS susceptibility from the HLA-C locus of the HLA complex (7) and from other loci, including IL-2Ra and IL-7Ra, (8), their contributory effects are small compared with the relatively strong association with the DRB1*1501 allele of HLA-DR. In patients of North European Caucasian origin, the HLA class II alleles that are most prevalent among MS patients come from the HLA-DR15 haplotype (or HLA-DR2, using old nomenclature from the period of serological

*Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel; †Human Disease Immunogenetics Group, Department of Infectious Diseases and Immunology, Imperial College, Hammersmith Hospital, London, United Kingdom; ‡Department of Immunology, Mayo Clinic, Rochester, MN 55905; and §Center for Brain Research, Department of Neuroimmunology, Medical University of Vienna, Vienna, Austria

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2 Address correspondence and reprint requests to Dr. Avraham Ben-Nun, Department of Immunology, Weizmann Institute of Science, P.O. Box 26, Rehovot 76000, Israel. E-mail address: avraham.ben-nun@weizmann.ac.il

3 Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; iNOS, inducible NO synthase; LNC, lymph node cell; MBP, myelin basic protein; MHCII, MHC class II; MOBP, myelin-associated oligodendrocytic basic protein; hMOBP, human MOBP; pMOBP, MOBP peptide; pHMOBP, human MOBP peptide; rMOBP, recombinant human MOBP; MOG, myelin oligodendrocyte glycoprotein; Mt. Mycobacterium tuberculosis; OSP, oligodendrocyte-specific protein; PLP, proteolipid protein; Tg, transgenic.

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MOBP was demonstrated in H-2b (18) and H-2s (19, 20) mice, and MOBP is expressed exclusively in CNS white matter and more. Immunoelectron microscopy (16) indicated that MOBP is a CNS myelin-specific protein that plays a role in stabilizing the myelin sheath (16). Northern blot analysis, in situ hybridization, immunohistochemistry, and immunoelectron microscopy (16, 17) indicated that MOBP is expressed exclusively in CNS white matter and more particularly throughout compact myelin, predominantly at the major dense lines of myelin (16). The encephalitogenic potential of MOBP was demonstrated in H-2b (18) and H-2s (19, 20) mice, and two separate studies of proliferative response to MOBP peptides (pMOBP) by BPL from MS patients and controls indicated that MOBP65–87 was the region most frequently recognized by MS patients T cells (18). Another study indicated that BPL obtained from relapsing/remitting MS patients mount a proliferative response to human MOBP, especially at 21–39 (21).

In this study, HLA-Tg mice expressing either the DRB1*1501 or DQB1*0602 heterodimers, as well as (DRB1*1501 × DQB1*0602),F1 double-Tg mice, were used to illuminate the relative potential contribution of each of these molecules, or their combination, to MOBP-associated pathogenic autoimmunity in MS patients with the “HLA-DR15 haplotype.” We show that whereas the HLA-DRB1*1501 transgenics are refractory to disease induction, the HLA-DQB1*0602-Tg mice are susceptible to EAE induction by hMOBP through pathogenic T cells reactive against MOBP15–36 and MOBP55–77 encephalitogenic epitopes. These data, which constitute a new “humanized” model of MS-like disease induced by hMOBP, also show that HLA-DQ6 can determine pathogenic antemyelin autoimmunity that leads to clinical EAE, MS-like pathology, and remarkable optic neuritis. These findings bear on the relative candidacy of the DRB1*1501 and DQB1*0602 alleles as genetic risk factors for MS susceptibility, as well as on devising immune-specific therapy for MS.

### Materials and Methods

**Mice**

HLA-DR15 (DRA1*0101:DRB1*1501) and (DRA1*0101,DRB1*1502)-Tg mice (MHCI Δ/ Δ) (referred to here as DRB1*1501-Tg and DRB1*1502-Tg, respectively) and HLA-DQ6 (DQA1*0103:DQB1*0601)-Tg mice (MHCI Δ/ Δ) (referred to here as DQB1*0601-Tg) were generated by F.S.D. (22). The HLA-DQ6 (DQA1*0102,DQB1*0602)-Tg mice (MHCI Δ/ Δ) (referred to here as DQB1*0602-Tg) were generated by D.M.A. Briefly, an HLA-DQA1*0102 cosmid clone with extensive 5' and 3' flanking sequences was cloned from a SuperCos library generated from genomic DNA made from the ROF-NL cell line (23). DQB1*0602 was cloned from a phagemid library generated from the DR15, DQ6 consanguineous cell line PCC. A positive clone was selected on the basis of positive screening with allele-specific primers as well as the presence of several hundred base pairs of 5' and 3' sequences. The vector-free inserts for the two genes were cojected and positive mice were screened by allele-specific PCR of tail biopsy DNA. HLA-DQ6 transgenic mice were crossed to the H-2B−/− line (24) and maintained thereafter as homozygous knockouts for H-2A.B. The lines were expanded at the animal facilities of the Weizmann Institute of Science (Rehovot, Israel), and progenies positive for the relevant transgenes by PCR were maintained. The Tg mice are homozygous with respect to the H-2A.B knockout. The mice are also homozygous for the HLA class II transgenes as interbreeding has produced no transgene-negative offspring for several generations, although a formal test cross with a wild-type partner was not conducted. The expression level of HLA-DRB1*1501 and HLA-DQB1*0602 on peripheral B cells from DRB1*1501- and DQB1*0602-Tg mice, respectively, is shown in supplemental Fig. S1.4

The HLA/DRB1*1501 × DQB1*0602 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 has approved the experiments, which were performed in accordance with its relevant guidelines and regulations.

### Recombinant human MOBP (rhMOBP) and hMOBP synthetic peptides

The rhMOBP gene corresponding to human OPRP2 (16) was constructed synthetically and expressed in Echerichia coli as described in Ref. 25 and the legend to supplemental Fig. S2. Human OPRP2 is the longer isoform (183 aa) of the human oligodendrocyte-specific proline-rich protein, OPRP, originally cloned and sequenced by Yamamoto et al. (16) and named human MOBP.

The amino acid sequences of synthetic overlapping peptides spanning hMOBP are listed in Table I. (All peptides (at least 80% purity) were synthesized in the laboratory of Prof. M. Fridkin, Department of Organic Chemistry, Weizmann Institute of Science, using the Fmoc technique with an automated peptide synthesizer (AMS422; Abimed).

#### Induction of EAE

Mice were injected s.c. at one site in the flank with 200 μl of emulsion containing 300 μg of hMOBP or 200 μg of peptide in CFA with 300 μg of Mycobacterium tuberculosis H37Ra (Mt) (catalog no. 3114-25; Difco Laboratories). Mice received 300 ng of pertussis toxin (catalog no. P-9452; 4 The online version of this article contains supplemental material. 55–77 QKYSEHFSIIIICCCPPTFTLNSKR
27–49 PFTTFLNSKREIVDRKYSICSG
37–60 RIYDKRYSICSGCFVQKXEEINDI
48–71 SGCFYQKKEDNIIICCACCQKTRRSTR
55–77 KEEDNIIICCACCQKTRRSTSRPQ
65–87 QKTTRTSRRAPKRCPQFPPFKQQAAP
75–97 PPRFPRQKQAPPAVPVVPPAPKRPR
106–125 PRSSPFRSEQPRSSPFRSEQ
138–162 PRFEPFPFFAKPRFPPCKSEQQPRSS
149–171 QRPFPKSEQQPRSSPSLRSPGASR
158–181 QPRSSPLRPQGASRGGSPVAKSRF
15–36 QDFPKSEQQPRSSPSLRSPGASR
55–77 KEEDNIIICCACCQKTRRSTSRAPQKP
Following the encephalitogenic challenge, mice were observed daily and an identical booster immunization on the flank 1 wk later (protocol 2). In some cases, as indicated, mice received a third immunization (protocol 1). In some cases, as indicated, mice received a third immunization (protocol 1).

The anti-hMOBP and anti-phMOBP Abs in sera sampled 30–40 days after immunization were measured by ELISA as described in Ref. 28. rhMOBP and synthetic peptides in PBS (10 μg/ml) were used for coating polystyrene, 96-well, polyvinyl chloride plates (Maxisorp; Nalge Nunc), and the normal mouse IgG1, IgG2a, IgG2b, or IgG3 (catalog nos. 1070-04, 1080-04, 1990-04, and 1100-04; Southern Biotechnology Associates) diluted 1/1000 and 100 μg/well hMOBP peptides (phMOBP) were used for coating polystyrene, and 100 μg/ml hMOBP peptides (phMOBP) were used for coating polystyrene, 96-well, polyvinyl chloride plates (Maxisorp; Nalge Nunc). The proliferative responses expressed as stimulation index (S.I.) were measured as described in Materials and Methods. Results are from pooled draining LNC from two mice immunized with each individual peptide and are representative of three independent experiments.

**Cytokine analysis**

IFN-γ, IL-4, and IL-10, were measured by ELISA according to the standard protocol from R&D Systems using a recombinant human TGF-β-sRII/Fc chimera as capture reagent (341-BR; R&D Systems) and biotinylated anti-human TGF-β1 Ab (BAF240; R&D Systems). Recombinant human TGF-β1 (240-B; R&D Systems) was used to construct the standard curve.

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 spinal cords that were sampled 35 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 Chemicals); T cells (CD3; Chemicon International) (19); inducible NO synthase (iNOS; Chemicon International); synaptophysin (Epinetics); mouse Ig (Amer-sham); and complement C9 (a gift from Dr. S. Fiedlens, University of Wales, Cardiff, U.K.). For staining, paraffin sections were pretreated with a steamer for 60 min. Bound primary Ab was detected with a biotin-avidin technique as previously described in detail (19).

**Results**

Mapping T cell epitopes of hMOBP in the DRB1*1501- and DQB1*0602-Tg mice

To define HLA-DR15 haplotype-related epitopes of hMOBP, the DRB1*1501- and DQB1*0602-Tg mice were immunized with each of the individual overlapping peptides spanning the whole hMOBP molecule (listed in Table I). Primed lymph node cells (LNC) were analyzed ex vivo for a recall proliferative response to variable concentrations of the immunizing peptide. Fig. 1A shows that phMOBP15–36, phMOBP48–71, phMOBP106–125, and phMOBP149–171 elicited in DRB1*1501-Tg mice a significant T cell response and therefore likely contain HLA-DR15-presented epitopes, with phMOBP15–36 and phMOBP48–71 stimulating the strongest HLA-DRB1*1501-restricted response. The DQB1*0602-Tg mice immunized with individual peptides showed significant T cell reactivity only to phMOBP15–36 and phMOBP55–77 (Fig. 1B), suggesting that for DQB1*0602-Tg mice, the hMOBP harbors only two major HLA-DQ6-associated T cell epitopes (or epitope clusters) located within the MOBP15–36 and MOBP48–77 regions.

In a similar manner, we also mapped the hMOBP T cell epitopes in DRB1*1502-Tg mice expressing the HLA-DRB1*1502 allele,
which differ from HLA-DRB1*1501 only in one aa residue (glycine for leucine substitution at position 86; Ref. 29), as well as in the HLA DRB1*0601 Tg mice expressing DQA1*0103;DQB1*0601 molecule as a non-HLA-DR15 haplotype-related control. As shown in Fig. 1C, only phMOBP15–36 and phMOBP37–60 were highly immunogenic for DRB1*1502-Tg, whereas in DQB1*0601-Tg mice only phMOBP27–49, phMOBP55–77, and phMOBP158–181 could elicit a significant T cell response (Fig. 1D).

These results suggest that the MOBP15–36, 37–60, 48–77, 106–125, and 149–181 regions of hMOBP encompass HLA-DR15-related T cell epitopes. Moreover, the consistent immunogenicity of MOBP15–36 in DRB1*1501-, DRB1*1502-, and DQB1*0602-Tg mice in several experiments suggests that the MOBP15–36 region contains an epitope of possible pathogenic relevance to this MS-related haplotype.

**Immunodominant T cell epitopes of rhMOBP in DRB1*1501- and DQB1*0602-Tg mice**

To validate the HLA-DR15-relevant epitopes that had been defined by immunization with the individual peptides and to distinguish which were immunodominant and which were cryptic, we immunized the HLA-DR- or HLA-DQ-Tg lines with rhMOBP that was prepared for this study (supplemental Fig. S2). Fig. S2C shows that the rhMOBP preparation was immunogenic for all of the different Tg lines tested, as the mice mounted a significantly higher specific T cell response to rhMOBP than that to the control nonrelevant recombinant protein rΔhPLP (recombinant soluble PLP deleted of putative hydrophobic domains).

Ex vivo analysis of the recall proliferative responses of rhMOBP-primed spleen cells derived from the different rhMOBP-immunized Tg lines to a panel of overlapping peptides spanning the hMOBP showed in several experiments a pattern of a codominant reactivity to phMOBP15–36 and phMOBP55–77 in DRB1*1501-Tg mice (Fig. 2A) and in DQB1*0602-Tg mice (Fig. 2B), whereas the DRB1*1502-Tg mice showed a clearly predominant reactivity against phMOBP15–36 only (Fig. 2C). The differences in epitope immunodominance in DRB1*1501-Tg mice upon immunization with the whole rhMOBP vs immunization with the overlapping peptides phMOBP48–71 and phMOBP55–77 (Figs. 1A and 2A) could be attributed to differences in the in vivo antigenic processing of the whole MOBP protein and the presentation of the naturally processed epitopes vs the presentation of synthetic peptides (30). The rhMOBP-primed spleen cells from the nonHLA-DR15-related (DQB1*0601) control Tg mice showed no significant proliferative responses to any of the hMOBP peptides. These results, together with those of epitope-mapping by peptide immunization (Fig. 1), identify MOBP15–36 and MOBP55–77 as the regions encompassing the epitopes of highest relevance to HLA-DR15 haplotype in the HLA-Tg mice.

**Epitope specificity of anti-hMOBP Abs in HLA-Tg mice**

Sera from different HLA-Tg lines immunized with rhMOBP/CFA that were collected 30 or 40 days postimmunization were analyzed for binding to overlapping hMOBP peptides. Fig. 3 shows that relatively high titers of Abs binding MOBP peptides could be detected only in the rhMOBP-immunized DQB1*0602-Tg mice (Fig. 3B), whereas anti-MOBP peptide Ab levels were relatively low in DRB1*1501-Tg mice (Fig. 3A) and under detection levels in the DRB1*1502-Tg mice (Fig. 3C). The Abs elicited in DQB1*0602-Tg mice showed no focused epitope specificity. Rather, the Abs were reactive against phMOBP1–23, phMOBP55–77, phMOBP65–87, phMOBP106–125, phMOBP138–162, and phMOBP149–171 (Fig. 3B). Interestingly, anti-hMOBP Abs could be detected only in the DQB1*0602-Tg mice, which were found to be the HLA-Tg line susceptible to induction of EAE (see below). The Abs against hMOBP-peptides were primarily of IgG1 and IgG2b isotypes, which are secreted by B cells that have undergone isotype switching regulated by Th2-type cells secreting IL-4 and Th1-type cells producing IFN-γ, respectively. The levels of IgG1 isotypes were at least 2-fold higher than those of IgG2b isotype, and Abs against the encephalitogenic epitope MOBP15–36 (see below) could not be detected.

**DQB1*0602- but not DRB1*1501-Tg mice are susceptible to hMOBP-induced EAE**

The various HLA-Tg mouse lines were immunized for the induction of EAE with the rhMOBP or hMOBP peptides that harbor a T cell epitope for each relevant HLA-Tg line (shown in Figs. 1 and
as well as with pMOBP15–36, which was previously shown to be encephalitogenic for H-2s mice (19, 20). The results summarized in Table II show that encephalitogenic inoculation with either immunogenic peptides or rhMOBP did not cause clinical EAE in DRB1*1501-, DRB1*1502-, or DQB1*0601-Tg mice. In contrast, encephalitogenic inoculation with phMOBP15–36 or phMOBP55–77 or with the whole rhMOBP caused the development of overt clinical EAE in DQB1*0602-Tg mice (Table II). The clinical course of EAE induced in HLA-DQB1*0602-Tg mice by phMOBP15–36 (Fig. 4A) or phMOBP55–77 (Fig. 4B) or by rhMOBP (not shown) is presented with clinical manifestations typical of classical EAE, with caudorostral ascending paralysis that developed 2–3 wk after immunization.

The DRB1*1501-Tg mice were resistant to induction of EAE regardless of whether phMOBP15–36, phMOBP55–77, or whole rhMOBP were used via protocol 1 or protocol 2 (with boost) of immunization. Why DRB1*1501-Tg mice (or DRB1*1502-Tg mice) did not develop EAE despite their ability to mount relatively strong T cell reactivity against pMOBP15–36 is puzzling. The human MOBP15–36 region differs from the autologous mouse MOBP15–36 in only one residue (Lys for Arg substitution at position MOBP36), and this one-residue difference is unlikely to be at the basis of EAE resistance of DRB1*1501-Tg mice. Indeed, the DRB1*1501-Tg mice were resistant also to EAE induction by an encephalitogenic inoculum of mouse serum was subtracted (0.01 ± 0.002).

MOBP15–36- and MOBP55–77-reactive T cells are Th1/Th17 in DQB1*0602-Tg and Th2 type in DRB1*1501-Tg mice

To further analyze the resistance of HLA-DRB1*1501-Tg mice to EAE induction by pMOBP15–36, we compared the cytokine secretion pattern associated with response to pMOBP15–36 and to phMOBP55–77 in HLA-DRB1*1501- and HLA-DQB1*0602-Tg mice. phMOBP15–36- and phMOBP55–77-primed LNC derived from HLA-DRB1*1501 or HLA-DQB1*0602-Tg mice were analyzed for secretion of Th1-/Th2-/Th17-type cytokines upon ex vivo stimulation with the priming peptide. The results presented in Fig. 5 show that the T cell response to phMOBP15–36 and to phMOBP55–77 in HLA-DQB1*0602-Tg mice was explicitly proinflammatory, with relatively high secretion of Th1/Th17 cytokines (IFN-γ and IL-17) and minimal secretion of Th2 cytokines (IL-4 and IL-10). These cytokine secretion profiles correlated well with the encephalitogenic potential of phMOBP15–36 and phMOBP55–77 in the HLA-DQB1*0602-Tg mice. In contrast, although pMOBP15–36 was immunogenic for HLA-DRB1*1501-Tg mice (Figs. 1 and 2), the relatively high T cell response to phMOBP15–36 was anti-inflammatory and associated with secretion of IL-4 and IL-10 and relatively low secretion of Th1/Th17 cytokines (IFN-γ and IL-17) (Fig. 5). The Th2 phenotype of the phMOBP15–36-reactive T cells corroborates the resistance of HLA-DRB1*1501-Tg mice to EAE induction by phMOBP15–36. phMOBP55–77 is nonencephalitogenic and poorly immunogenic for HLA-DRB1*1501-Tg mice, and except for a relatively low level of IL-10, the secretion of other cytokines in response to pMOBP55–77 was very low. Analysis of the cytokine secretion profile of hMOBP-primed LNC derived from HLA-DRB1*1501- or HLA-DQB1*0602-Tg mice showed similar Th1 patterns of cytokine secretion upon stimulation by pMOBP15–36 and pMOBP55–77 of HLA-DQB1*0602-derived hMOBP-primed LNC and a similar Th2 pattern upon stimulation by pMOBP15–36 of HLA-DRB1*1501-derived hMOBP-primed LNC (data not shown). It is well worth

FIGURE 3. Epitope specificity of anti-hMOBP Abs in HLA-Tg mice. HLA-DRB15- and HLA-DQ6-Tg mice were immunized with hMOBP/CFA for EAE induction (protocol 1) as described in Materials and Methods and blood was sampled on day 30 (mice 1–3) or day 40 (mouse 4) after immunization. A–D, Mapping the hMOBP B cell epitopes in HLA-DR15-Tg mice (DRB1*1501 (A) and DRB1*1502 (C)) and HLA-DQ-Tg mice (DQB1*0602 (B) and DQB1*0601 (D)). A panel of overlapping peptides spanning the hMOBP was used to measure specific reactivity to peptides by ELISA. Serum was diluted to 1/100 with PBS. E, Isotype determination for Abs reactive to phMOBP in DQB1*0602-Tg mice. Values represent reactivity of hMOBP-immunized sera from which background reactivity of normal mouse serum was subtracted (0.01 ± 0.002).
noting here that these DRB1*1501-Tg mice could mount pathogenic Th1/Th17 responses against mouse MOG35–55, and were susceptible to induction of EAE by MOG35–55/CFA (data not shown). Whether the resistance to pMOBP15–36-induced EAE is also associated with the induction of regulatory T cells is not yet known. However, the low levels of TGFβ secreted upon recall proliferative response to pMOBP15–36 in either HLA-Tg line may not favor this possibility.

**T cell autoimmunity against rhMOBP in HLA-(DRB1*1501 × DQB1*0602)F1 double-Tg mice**

The HLA-DR15 haplotype that is the most prevalent among Caucasian populations with MS comprises the class II allelic genes DRB1*1501 and DQB1*0602 in strong linkage disequilibrium. This makes dissociation of the relative contribution of each allele to the increased disease susceptibility extremely difficult. Their coexistence, however, may each influence other allele-restricted T cells against myelin proteins and thereby influence epitope specificity and the net pathogenic antimyelin autoimmunity. We thereforecrossed the DRB1*1501-Tg mice with DQB1*0602-Tg mice to generate HLA-(DRB1*1501 × DQB1*0602)F1 double-Tg mice (HLA(DR15 × DQ6) double-Tg mice) and immunized the mice with rhMOBP. The rhMOBP-primed spleen cells were analyzed ex vivo for recall proliferative responses against rhMOBP as well as against a panel of overlapping peptides spanning the MOBP molecule. Fig. 6 shows the immunogenicity of rhMOBP in these mice; the rhMOBP-primed spleen cells from HLA(DR15 × DQ6) double-Tg mice showed a significant and specific proliferative response to rhMOBP, as the spleen cells did not proliferate in the presence of the nonrelevant recombinant myelin protein rΔHPLP (deleted of putative hydrophobic domains), which was expressed and purified in the same manner as the rhMOBP.

### Table II. Active EAE induction with hMOBP or MOBP peptides in HLA-Tg mice

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<td></td>
<td>pMOBP75–97</td>
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<sup>a</sup> EAE was induced by s.c. injection at the flank with 200 μl of CFA emulsion containing 200 μg of hMOBP or phMOBP and mice received 300 ng of pertussis toxin immediately and 48 h after immunization (protocol 1).

<sup>b</sup> Mice received also an identical booster immunization at the other side of the flank, one week later (protocol 2).

<sup>c</sup> EAE was induced by s.c. injection at the flank with only 100 μl of CFA emulsion containing 100 μg of phMOBP and mice received 300 ng of pertussis toxin immediately and 48 h after immunization.

### Figure 4.

**A.** Clinical course of active EAE induced in DQB1*0602-Tg mice. A, phMOBP15–36-induced EAE by s.c. injection at the flank with 200 μl of CFA emulsion containing 200 μg. Immediately after immunization and 48 h later mice were injected i.v. with 300 ng of pertussis toxin (protocol 1). B, Two experiments of phMOBP55–77-induced EAE are shown. In one experiment (filled circles) EAE was induced as in A but with phMOBP55–77. In the other experiment (open circles) EAE was also induced as in A but with a lower amount of inoculum (100 μl of CFA emulsion containing 100 μg of phMOBP55–77). The asterisk (*) indicates that the experiment was terminated and mice were sacrificed for pathological examination. Mean clinical severity includes mice that did not develop EAE (with 0 clinical severity). I, Incidence.
vivo analysis of recall proliferative responses by rhMOBP-
primed spleen cells to the overlapping peptides indicated that
phMOBP15–36, phMOBP27–49, phMOBP48–71, and phMOBP75–97 contain hMOBP-T cell epitopes for the
HLA(DR15/H11003/DQ6) double-Tg mice (Fig. 6). As phMOBP48–71 and phMOBP55–77 overlap with a relatively long stretch of
amino acid sequence, it is highly likely that they share the same T
cell epitope(s).

A comparison of the epitope specificity of the hMOBP-reactive
T cells from the HLA-(DRB1*1501/DQB1*0602)F1 double-Tg mice with that of hMOBP-reactive T cells from the parental
HLA-(DRB1*1501/DQB1*0602)F1 double-Tg mice revealed that the
HLA-(DRB1*1501/DQB1*0602)F1 double-Tg mice were susceptible to EAE induced by hMOBP and by phMOBP15–36 and phMOBP55–77 that were encephalitogenic for the parental
DQB1*0602-Tg mice. In contrast, phMOBP27–49, phMOBP48–71,
and phMOBP75–97, which were also stimulatory for hMOBP-reactive T cells in HLA(DR15/H11003/DQ6) double-Tg mice (Fig. 6),
were not encephalitogenic (Table II). Fig. 7 shows the clinical
course of EAE in the HLA-DR15 double-Tg mice immunized with
rhMOBP (Fig. 7A), phMOBP15–36 (Fig. 7B), or phMOBP55–77 (Fig. 7C). As can be seen, EAE induced by phMOBP55–77 was
significantly more severe than EAE induced by either hMOBP or
phMOBP15–36, suggesting that MOBP55–77 encompasses the ma-
jor encephalitogenic epitope of hMOBP for mice coexpressing the
MS-relevant alleles of the HLA-DR15 haplotype. Because
phMOBP55–77 and pMOBP15–36 are encephalitogenic only in the
parental DQB1*0602 Tg mice, it is likely that their encephalito-
genic potential is associated with DQB1*0602.

FIGURE 6. Epitope specificity of hMOBP-primed T cells derived from
HLA-(DRB1*1501/DQB1*0602)F1 double-Tg mice. A, Immunogenicity of hMOBP in HLA-(DRB1*1501/DQB1*0602)F1 double-Tg mice. Spleen cells from mice that were immunized 14 days earlier with 200 μg of hMOBP in CFA for EAE induction (protocol 2) were analyzed ex vivo for their recall proliferative response to hMOBP or the purified protein
derivative (PPD) or to rhPLP (ΔhPLP; 5 μg/ml) as a control nonrelevant recombinant protein that is deleted of its putative hydrophobic domains and expressed and purified similarly as hMOBP. B, Epitope-specificity. HLA-(DRB1*1501/DQB1*0602)F1 double-Tg mice were immunized with 200 μg of hMOBP in CFA and received pertussis toxin (300 ng) imme-
diately after immunization and 48 h later. On day 14 after immunization the
spleen cells were analyzed ex vivo for their recall proliferative response to
overlapping peptides spanning the hMOBP. Spleen cells were cultured in triplicates in the absence or presence of peptides at the indicated concen-
trations and incubated for 72 h. [3H]Thymidine was added for the last 16 h.

S.I., Stimulation index.

HLA(DR15 × DQ6) double-Tg mice are susceptible to
hMOBP-induced EAE

The HLA(DR15 × DQ6) double-Tg mice were immunized with
CFA emulsion containing rhMOBP or peptides that were defined
above as encompassing hMOBP epitopes for the double-Tg mice. As shown in Table II, the HLA(DR15 × DQ6) double-Tg mice
were susceptible to EAE induced by hMOBP and by pMOBP15–36 and pMOBP55–77 that were encephalitogenic for the parental
DQB1*0602-Tg mice. In contrast, pMOBP27–49, pMOBP48–71, and pMOBP75–97, which were also stimulatory for hMOBP-re-
active T cells in HLA(DR15 × DQ6) double-Tg mice (Fig. 6),
were not encephalitogenic (Table II). Fig. 7 shows the clinical
course of EAE in the HLA-DR15 double-Tg mice immunized with
rhMOBP (Fig. 7A), phMOBP15–36 (Fig. 7B), or phMOBP55–77 (Fig. 7C). As can be seen, EAE induced by phMOBP55–77 was
significantly more severe than EAE induced by either hMOBP or
pMOBP15–36, suggesting that MOPBP55–77 encompasses the ma-
jor encephalitogenic epitope of hMOBP for mice coexpressing the
MS-relevant alleles of the HLA-DR15 haplotype. Because
pMOBP55–77 and pMOBP15–36 are encephalitogenic only in the
parental DQB1*0602 Tg mice, it is likely that their encephalito-
genic potential is associated with DQB1*0602.

MS-like pathology and optic neuritis induced by hMOBP in the
HLA Tg mice

Histopathology of EAE was essentially similar between HLA-
DQB1*0602-Tg and HLA-(DRB1*1501 × DQB1*0602)F1 double-Tg mice. It consisted of perivascular inflammatory infiltrates
that were most pronounced in the spinal cord (Fig. 8, Aa, Ad, and
FIGURE 7. Clinical course of MOBP-induced EAE in (DRB1*1501 × DQB1*0602)F1, double-Tg mouse. Mice were injected s.c at the flank with 200 µl of emulsion containing 300 µg of Mt and 200 µg of whole hMOBP (A), pMOBP15–36 (B), or pMOBP55–77 (C). Pertussis toxin (300 ng) was injected i.v. immediately and 48 h after immunization. I, Incidence; M, mortality.

FIGURE 8. Histopathology of EAE in (DRB1*1501 × DQB1*0602)F1 double-Tg mouse. Samples were taken on day 35 after immunization. Shown are the spinal cord (A), the cerebellum (B), and the optic nerve (C). The sections were stained with H&E (a), Luxol fast blue (b), Bielschowsky silver impregnation for axons (c) and by immunocytochemistry for CD3 (d), Mac-3 (e), and iNOS (f). Profound inflammation (a, d, and e) is associated with confluent demyelination (b) and profound axonal injury (c). The inset in Bd shows a higher magnification of T cell infiltrates in the cerebellum, and the inset in Cc shows a high magnification of a damaged axon in the optic nerve. Inflammation is associated with profound expression of iNOS in macrophages and microglia (Cf).

Ae) and the optic nerves (Fig. 8, Ca, Cd, and Ce) but also affected, with lower incidence, the cerebellar white matter (Fig. 8, Ba, Bd, and Be) and the brain stem (including medulla oblongata, mesencephalon, and basal ganglia (data not shown). Profound inflammation was also seen in the meninges of brain and spinal cord. Inflammatory infiltrates were rare in the periventricular white matter and the cerebral cortex. Inflammatory infiltrates were mainly composed of CD3-positive T cells (Fig. 8, Ad and Bd) and macrophages Fig. 8, Ae and Be). In addition, profound microglial activation was seen in affected brain and spinal cord regions (Fig. 8, Ae and Be). Inflammation was associated with profound iNOS expression in macrophages and microglia (Fig. 8 Cf).

Inflammation was associated with a variably extent of demyelination and acute axonal injury (Fig. 8, a–c for A–C). The highest extent of demyelination was seen in the optic nerves followed by the spinal cord, where confluent demyelinated plaques were found. Within inflamed areas the blood brain barrier was impaired, leading to diffuse leakage of immunoglobulins into the tissue. However, no precipitation of immunoglobulins or complement C9 was seen on myelin or other tissue elements (data not shown).

Discussion

The studies presented here describe a new model of “humanized” EAE induced by MOBP in HLA-Tg mice expressing the DRB1*1501 and DQB1*0602 alleles of HLA-DR15, the most prominent HLA haplotype in North European Caucasian MS. The induction of EAE associated with demyelination, axonal loss, and optic neuritis in the HLA-Tg mice expressing alleles of the HLA-DR15 haplotype further emphasizes the relevance of autoimmunity against MOBP to the pathogenesis of MS, in addition to the other more commonly analyzed target myelin proteins such as MBP, PLP, and MOG. Furthermore, the demonstration that MOBP is encephalitogenic in HLA-DQB1*0602-Tg mice and HLA-DR15 × DQ6 double-Tg mice, but not HLA-DRB1*1501-Tg mice, offers a potential mechanism of the HLA-DQB1*0602 allele in conferring genetic susceptibility to autoimmunity in humans. These results are the first to demonstrate pathogenic antimmunoglobulin autoimmunity mediated through HLA-DQB1*0602 presentation. This assumes particular relevance in view of the long preoccupation of MS immunologists with HLA-DRB1*1501 as the key Ag-presenting molecule implicated by MS immunogenetics (15).

Although recent genome-wide association MS studies indicate that the alleles of genes encoding IL-2Rα and IL-7Rα are significantly associated with disease susceptibility (8), they also confirm that the genes of the HLA-DR15 haplotype show the strongest genetic association with susceptibility to MS (8, 31). MS genetic studies that focus on the HLA association with MS also suggest that DRB1*1501 is the primary risk factor for increased susceptibility to MS (9, 32). However, the strong linkage disequilibrium of HLA class II genes in humans imposes difficulties in discerning the relative contribution of the DRB1*1501, DRB5*0101, and the DQB1*0602 products of the HLA-DR15 haplotype to MS predisposition.

There has been something of a dichotomy in studies of MS T cell immunogenetics, in terms of which the class II heterodimers...
encoded by the HLA-DR15 haplotype are considered to be functionally implicated in both mechanism and susceptibility. Functional T cell studies looking at T cell lines and clones have focused almost entirely on myelin epitope presentation through HLA-DR and, in particular, by the DRA1*0101/DRB1*1501 pair (33, 34), although there have been occasional reports of HLA-DQ6-restricted T cells clones from MS patients, including cells specific for MBP 85–99 (35, 36). Lack of clarity as to which MHCII peptide specificities are relevant to pathogenesis has relevance to many areas of endeavor in MS research, from analysis and immune-specific targeting of pathogenic TCRs (37) to peptide immunotherapies (38) and programs tracking patients’ responses during disease (39). Although most MS genetics studies in North European Caucasian populations suggest HLA-DRB1*1501 as the genetic risk factor in MS (40–44), other genetic studies in unique populations suggest that HLA-DQ alleles may also be a risk factor. Spurkland, Thorsby, Vartdal and coworkers studied the specific HLA-DR and -DQ alleles of 181 Norwegian MS patients (12). Patients were identified who carried DQB1*0602 or 0603 without DRB1*1501, but none who were DRB1*1501 without DQB1*0602 (12). Related observations were made in a relatively small sample of Hong Kong Chinese patients with MS. In that population, DR15 is expressed without DQB1*0602 and DQB1*0602 without DR15, so that one can ask whether either or both of the dissociated genes appear to confer an enhanced risk. It was found that the enhanced risk was associated with DQB1*0602 (13). A similar case has been made for susceptibility in Afro-Brazilians, where the frequency of DQB1*0602 among patients is higher than that for the main DR15 allele, DRB1*1503, in that unique population (45). A caveat here is the analysis of HLA-DR and -DQ associations conducted in a large cohort of African American MS patients (14). Because African populations show greater haplotype diversity than Europeans and also show distinct patterns of linkage disequilibrium, it was possible to appraise separately the relative contributions of HLA-DR and -DQ sequences to susceptibility. In this example, a selective association with HLA-DRB1*1501 and not with HLA-DQB1*0602 was identified (14).

HLA-Tg mice offer an important tool for illuminating the association of disease susceptibility with the specific gene products of the HLA-DR15 haplotype. Studies with HLA class-II Tg mice have previously demonstrated HLA-DR-dependent disease induced by MBP, PLP, and MOG (42, 46–49). In our study, HLA-DRB1*1501- and HLA-DQB1*0602-Tg mice and HLA-DRB1*1501 × DQB1*0602F1 double-Tg mice were used to discern the relative contribution of the class-II heterodimers, either individually or in combination, to the development of MOBP-associated pathogenesis. Our results show that, unlike previously reported studies with MBP-, PLP-, or MOG-induced EAE, pathogenic autoimmunity against MOBP was dependent on HLA-DQB1*0602 rather than HLA-DRB1*1501. This is in striking contrast with human and transgenic mouse studies suggesting a protective role for HLA-DQ6. The closely related allele DQB1*0601, most typically found in Asian populations, has been reported in two human studies to be associated with protection from MS (50, 51). In accordance with those findings, studies by one of the authors of this manuscript (22, 52) argued that the presence of HLA-DQB1*0601 could exert an epistatic protective effect on HLA-DR-dependent, antimyelin autoimmunity. Thus, while HLA-DRB1*1501 Tg mice were susceptible to MOG-induced EAE, the HLA-DRB1*1502 × DQB1*0601 double-Tg mice were resistant (22). The protective effect of HLA-DQ6(DQA1*0103/DQB1*0601) was reported in more detail in PLP-induced EAE, in which HLA-DR3(DRB1*0301)-Tg mice were susceptible to PLP91–110-induced disease, whereas the HLA-DR3(DRB1*0301) × DQ6(DQB1*0601) double-Tg mice were protected (52). In this study, using the Caucasian MS-associated allelic genes we show the opposite scenario for the role of the HLA-DQ6 in antimyelin autoimmunity in HLA-(DR15 × DQ6)-Tg mice. Thus, the HLA-DQB1*0602 gene product determined genetic susceptibility to MOBP-induced EAE, the HLA-DRB1*1501 × DQB1*0602F1 double-Tg mice were as susceptible to MOBP-induced EAE as their parental HLA-DQB1*0602-Tg mice for both encephalitogenic MOBP epitopes (MOBP15–36; MOBP55–77), and the presence of the HLA-DRB1*1501 gene product in the HLA-DRB1*1501 × DQB1*0602F1 double-Tg mice did not impose any immunomodulatory effect.

Many myelin epitopes encephalitogenic for genetically susceptible wild-type mice have been found to be encephalitogenic also in HLA-DR15-Tg mice (46, 48, 49) and often T cells against these epitopes were also observed in MS patients (53–55), suggesting that the pathogenic responses modeled here have value for the analysis of events underpinning human disease and the design of therapeutics. In this respect pMOBP15–36, which is encephalitogenic in SJL/J mice (19, 20), encompasses an epitope recognized by MS patients’ PBL (19), and predicted to contain an HLA-DR15 epitope (19), was expected to be encephalitogenic in HLA-DRB1*1501-Tg mice. However, pMOBP15–36 was encephalitogenic only in transgensics expressing HLA-DQB1*0602. Why the HLA-DRB1*1501-Tg mice were resistant to MOBP15–36-induced EAE is puzzling, particularly in view of the findings that both HLA transgensics could mount T cell responses to MOBP15–36. The likely explanation was revealed by cytokine analysis of responses in the different genetic contexts; immunization with pMOBP15–36 (or with whole hMOBP; data not shown) elicited a largely Th2-type profile in HLA-DRB1*1501-Tg MOBP-reactive T cells (high IL-4 and IL-10 with very low IFN-γ and IL-17). In contrast, immunization of DQB1*0602 Tg mice with pMOBP15–36 or pMOBP55–77 elicited responses mostly of the Th1/Th17-type with negligible Th2 cytokines. Thus, the use of the individual Tg lines to dissect the HLA-DR15 haplotype-disease association offers insights into the ability of peptide presentation through different HLA class II products to skew Th cell cytokine polarization with accompanying implications for pathogenesis.

EAE induced by pMOBP55–77 tended to be more pronounced than that induced by pMOBP15–36 in the HLA-(DRB1*1501 × DQB1*0602)F1 double-Tg mice. Nevertheless, both immunogens caused clinical EAE associated with perivascular and parenchymal infiltrates, widespread demyelination, axonal loss, and optic neuritis with a high degree of demyelination in the optic nerve that is of variably intensity and correlates with disease severity. The pathology was essentially similar between HLA-DQB1*0602 and HLA-DRB1*1501 × DQB1*0602F1 double-Tg mice, and the pathological manifestations were highly reminiscent of MS. The pathological manifestations of MOBP-induced EAE in the HLA-Tg mice were also similar to those of MOBP-induced EAE in the wild-type SJL/J mice mediated by CD4+ T cells specific for pMOBP15–36 (19, 20). Although not shown, the MOBP15–36- and MOBP55–77-reactive cells derived from HLA-DQB1*0602-Tg mice were CD4+ T cells and were DQ6-restricted, as evident from the blocking of their peptide-specific proliferative response in vitro with an anti-CD4 mAb or a relevant anti-DQ, but not with anti-DR mAb.

Overall, this new “humanized” model of MS-like disease provides the first evidence of pathogenic HLA-DQ-presented antimyelin autoimmunity and implicates autoimmunity against MOBP in the pathogenesis of MS. It also, for the first time, offers a rationale for the HLA-DQB1*0602 association with MS. In view of the likelihood of complex patterns of myelin autoimmunity in MS that
may encompass responses to MBP, PLP, MOG, MOBP, OSP, or other as yet undefined CNS components, it is highly likely that the functionally implicated HLA-DR/DQ products might differ for autoreactivity against different myelin target Ags/epitopes. Further work using complementary studies of reductionist transgenic models with the more complex analysis of patient responses will be necessary to elucidate these patterns, particularly if T cell specific immunotherapies are to be considered.

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Disclosures
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References