Multiple sclerosis

Molecular mimicry of an antimyelin HLA class I restricted T-cell receptor

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ABSTRACT

Objective: To identify target antigens presented by human leukocyte antigen (HLA)–A*02:01 to the myelin-reactive human T-cell receptor (TCR) 2D1, which was originally isolated from a CD8+ T-cell clone recognizing proteolipid protein (PLP) in the context of HLA-A*03:01, we employed a new antigen search technology.

Methods: We used our recently developed antigen search technology that employs plasmid-encoded combinatorial peptide libraries and a highly sensitive single cell detection system to identify endogenous candidate peptides of mice and human origin. We validated candidate antigens by independent T-cell assays using synthetic peptides and refolded HLA:peptide complexes. A molecular model of HLA-A*02:01:peptide complexes was obtained by molecular dynamics simulations.

Results: We identified one peptide from glycerolphosphatidylcholine phosphodiesterase 1, which is identical in mice and humans and originates from a protein that is expressed in many cell types. When bound to HLA-A*02:01, this peptide cross-stimulates the PLP-reactive HLA-A3-restricted TCR 2D1. Investigation of molecular details revealed that the peptide length plays a crucial role in its capacity to bind HLA-A*02:01 and to activate TCR 2D1. Molecular modeling illustrated the 3D structures of activating HLA:peptide complexes.

Conclusions: Our results show that our antigen search technology allows us to identify new candidate antigens of a presumably pathogenic, autoreactive, human CD8+ T-cell-derived TCR. They further illustrate how this TCR, which recognizes a myelin peptide bound to HLA-A*03:01, may cross-react with an unrelated peptide presented by the protective HLA class I allele HLA-A*02:01. *Neurol Neuroimmunol Neuroinflamm* 2016;3:e241; doi: 10.1212/NXI.0000000000000241

GLOSSARY

58-2D1-CD8-sGFP = 58$\alpha^-\beta^-$ T hybridoma cells expressing TCR 2D1, human CD8$\alpha\beta$ molecules, and sGFP under the control of nuclear factor of activated T cells; **58-B7-CD8-sGFP** = 58$\alpha^-\beta^-$ T hybridoma cells expressing TCR B7, human CD8$\alpha\beta$ molecules, and sGFP under the control of nuclear factor of activated T cells; **APC** = antigen-presenting cells; **COS-7-A2** = COS-7 cells expressing HLA-A*02:01; **COS-7-A3** = COS-7 cells expressing HLA-A*03:01; **DMXL2** = Dmx-like-2; **EAE** = experimental autoimmune encephalomyelitis; **EML5** = echinoderm microtubule associated protein-like 5; **GPCPD1** = glycerolphosphatidylcholine phosphodiesterase 1; **HLA** = human leukocyte antigen; **HLA-A2** = HLA-A*02:01; **HLA-A3** = HLA-A*03:01; **IL** = interleukin; **Met** = methionine; **MHC** = major histocompatibility complex; **MS** = multiple sclerosis; **mTECs** = medullary thymic epithelial cells; **NCAN** = Neurocan; **NFAT** = nuclear factor of activated T cells; **PECP** = plasmid-encoded combinatorial peptide; **PLP** = proteolipid protein; **RMSD** = root mean square deviation; **PLP** = proteolipid protein; **TAX** = T-lymphotrophic virus-2 protein; **TCR** = T-cell receptor; **VMD** = visual molecular dynamics.

Multiple sclerosis (MS) is a chronic, presumably autoimmune disease of the CNS.[1,2] As with other autoimmune diseases, the triggers of the autoimmune reaction are unknown. However, cross-reactivity between autologous self-antigens and microbial non-self-antigens, termed molecular mimicry, has long been considered as candidate mechanism.[3–5] This holds especially

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Funding information and disclosures are provided at the end of the article. Go to Neurology.org/nn for full disclosure forms. The Article Processing Charge was paid by the authors.

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for T cells, which recognize short peptides bound to antigen-presenting major histocompatibility complex (MHC) molecules. Here, 3 types of mimicry are conceivable: first, one T-cell receptor (TCR) recognizes different peptides presented by the same autologous MHC molecule; second, one TCR cross-reacts with MHC:peptide complexes where both MHCs and peptides are different, but the MHCs are both autologous; and third, one TCR crossreacts with peptides presented by allogeneic (non-self) MHC molecules, termed alloreactivity.[6]

Myelin-reactive CD4+ T lymphocytes are considered to be the major effector cells in experimental autoimmune encephalomyelitis (EAE), and by extrapolation, also in MS.[7,8] For CD4+ T cells, several examples of mimicry between myelin autoantigens and microbial antigens,[9–11] as well as mimicry between a myelin and neuronal autoantigen,[12] have been described. However, relatively little is known about mimicry reactions involving CD8+ T cells,[13] although CD8+ T cells predominate in MS lesions.[14–16] In addition, they show signs of clonal expansion, clonal persistence, and pervasiveness, suggesting that many brain-infiltrating CD8+ T cells recognize shared antigens.[16–18]

We studied molecular mimicry that involves 2 different human leukocyte antigen (HLA) class I molecules using one of the few well-characterized human myelin-reactive CD8+ TCRs as a paradigm. This TCR, termed 2D1, was isolated from a patient with MS.[19] TCR 2D1 has interesting properties in that it recognizes a peptide from proteolipid protein (PLP[45-53]) in context of the HLA class I molecule HLA-A*03:01 (HLA-A3). Furthermore, evidence obtained with a humanized TCR and HLA multiple-transgenic mouse model indicated that this TCR also might recognize unknown peptides in context of HLA-A*02:01 (HLA-A2).[20] Of note, HLA-A2 is thought to have a protective effect on MS.[21] To identify candidate antigens that might be recognized by this PLP-reactive, HLA-A3-restricted TCR in context of the protective HLA-A2 molecule, we applied our method for the unbiased identification of T-cell target epitopes.[22] We identified a peptide from glycerolphosphatidylcholine phosphodiesterase 1 (GPCPD1[14-22]) that strongly activated TCR 2D1 when presented by HLA-A2. Using biochemical analysis and molecular dynamics calculations, we provide a model of the HLA-A2:GPCPD1(14-22) complex that cross-reacts with HLA-A3:PLP(45-53).

METHODS

Standard protocol approvals, registrations, and patient consents. The study was approved by the ethics committee of the medical faculty of the Ludwig-Maximilian-University Munich.

Synthetic peptides. The peptides PLP(45-53): (KLIETYFSK), TAX(11-19): (LLFGYPVYV), Dmx-like-2 (DMXL2) DMXL2 (813-820): (LIGEVFNI), DMXL2(812-820): (KLIGEVFNI), M-DMXL2(813-820): (MLIGEVFNI), echinoderm microtubule associated protein-like 5 (EML5) EML5(997-1004): (MEGEVWGL), EML5(996-1004): (HMEGEVWGL), M-EML5(997-1004): (MMEGEVWGL), GPCPD1(15-22): (LPGEVFAI), GPCPD1(14-22): (LLPGEVFAI), M-GPCPD1 (15-22): (MLPGEVFAI), Neurocan (NCAN) NCAN(257-264): (LGGEVFYV), NCAN(256-264): (ELGGEVFYV), and M-NCAN(257-264): (MLGGEVFYV) were purchased from Thermo Fisher (Unterelchingen, Germany) at purities >95%, except TAX(11-19) and GPCPD1(15-22) used for the refolding experiment, which were 85% pure.

Plasmids and generation of transfected T hybridoma and COS-7 cells. The T hybridoma cells $58\alpha^{-}\beta^{-23}$ expressing human CD8 α- and β-chains and the α- and β-chains of TCR 2D1[19] are described.[20] 2D1 is a dual-α-TCR[24] that expresses a Vβ15.1Jβ2.7-chain and 2 α-chains, Vα2Jα21 and Vα7Jα11.[20] These cells were used throughout except for one experiment, where we used $58\alpha^{-}\beta^{-}$ cells transfected with the β-chain and only one of the α-chains.[20] TCR B7, which recognizes a peptide from human T-lymphotrophic virus-2 TAX protein (TAX[11-19]) in the context of HLA-A2,[25] was cloned and transfected analogously. TCR transfectants were stably supertransfected with pcDNA-NFAT-sGFP plasmid.[22] These cells are termed 58-2D1-CD8-sGFP and 58-B7-CD8-sGFP, respectively.

COS-7 cells that stably express HLA-A2 (termed COS-7-A2 cells) are described.[22] COS-7 cells that stably express HLA-A3 (COS-7-A3) were generated analogously. HLA-A2 expression was analyzed by flow cytometry using the FITC-labeled antibody BB7.2 (Proimmune, Oxford, UK) and by activation of 58-B7-CD8-sGFP cells incubated with TAX(11-19). Since no HLA-A3-specific antibody is available, COS-7-A3-expressing clones were incubated with the peptide PLP(45-53) and analyzed by activation of 58-2D1-CD8-sGFP cells.

All cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany).

Construction of plasmids coding for plasmid-encoded combinatorial peptide (PECP) libraries and peptides. The construction of PECP libraries and of plasmids expressing defined peptides is described.[22] Here we used an 8 amino acid library (termed $8X^{8L}$) with randomized amino acids at positions 1 to 7 encoded by the nucleotides NNK (N = A, T, C, or G; K = G or T) and a fixed leucine in position 8 that serves as binding anchor to HLA-A2.[26] Our library contained 3.94×10^{8} independent clones of 1.3×10^{9} theoretically possible clones. The

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library and the oligonucleotides encoding defined peptides (Metabion, Martinsried, Germany) were inserted into the recipient plasmid pcDNArc and transfected into COS-7-A2 or COS-7-A3 cells.[22]

Identification of mimotopes and bioinformatic prediction of candidate antigens. The assay for identification of mimotopes by PECP libraries is described.[22] Briefly, to analyze TCR activation by plasmid-encoded peptides, COS-7-A2 or COS-7-A3 cells were seeded in cell culture plates and transiently transfected with peptide-encoding plasmids. With the $8X^{8L}$ library, we tested 2.5×10^7 COS-7-A2 cells. After 48 hours, 58-2D1-CD8-sGFP or 58-B7-CD8-sGFP T hybridoma cells were added. Sixteen hours later, T hybridoma cell activation was monitored by measurement of secreted mouse interleukin (IL)-2 by mouse IL-2 ELISA (eBioscience, Frankfurt, Germany) or by fluorescence microscopy for sGFP expression.[22]

To analyze TCR activation by synthetic peptides, 3×10^4 COS-7-A2 or COS-7-A3 cells were seeded in 96-well flat-bottom cell culture dishes. After 4 hours, peptides were added to a final concentration of 0.5 mM. After 30 minutes, 3×10^4 58-2D1-CD8-sGFP or 58-B7-CD8-sGFP cells were added and T-cell activation was observed 16 hours later as described above.

Bioinformatic searches were initially based on the 3 detected mimotopes and then refined based on recognized and nonrecognized candidate peptides. The program PROPHECY from the EMBOSS suite[27] was used to create a frequency matrix. The tool PROFIT[27] was then employed with this matrix to scan the fasta files from the Uniprot database. The searched taxa-specific protein sequence files were downloaded from UniprotKB[28] *Mus musculus* (10090) and *Homo sapiens* (9606). The results were then annotated using a python script and potential targets were further prioritized by us.

Refolding of HLA-A2:β2m:peptide complexes and T-cell activation assay. The nucleotide sequences coding for β_2m and the HLA-A2 heavy chain (amino acids 1-275), which was extended for a linker (GS) and a BirA biotinylation site, were inserted into the plasmid pET-21c(+), expressed, and purified as described.[29,30] Gel filtration was replaced by dialysis steps against 25 mM MES, pH 6.0, 8 M urea, 10 mM EDTA, 2 mM dithiothreitol (Sigma, Deisenhofen, Germany) and then against the same buffer containing 0.1 mM dithiothreitol. HLA-A2, β2m, and peptides were refolded to heterotrimeric complexes by dilution at a final molar ratio of 1:1:5.[29] The complexes were labeled with biotin and concentrated by ultrafiltration.[29]

For activation of 58-2D1-CD8-sGFP cells, Pierce high-sensitivity streptavidin-coated plates (Thermo Fisher Scientific, Waltham, MA) were washed 3 times with phosphate-buffered saline and incubated with 1.8 μg refolded HLA-A2:β2m:peptide complexes for 30 minutes at room temperature. After removal of the supernatant, 58-2D1-CD8-sGFP T hybridoma cells were added and T-cell activation was analyzed by fluorescence microscopy and ELISA as described above.

Molecular dynamics simulation. The X-ray-based model of HLA-A2 PDB-ID:1OGA[31] was used as a template for homology modeling and subsequent molecular dynamics simulation. HLA α- and β2m-chains with the bound peptide flu(58-66) were extracted and the peptide sequence was mutated to the nonamers LLPGEVFAI and MLPGEVFAI. To model the octamer complex with HLA-A2, we used the structure of HLA-A2 with the peptide TAX8[32] PDB-ID:1DUY and mutated the bound peptide to the octamer LPGEVFAI. The homology modeling was carried out by Iterative Reduction of Conformational Space (IRECS).[33]

Molecular dynamics simulations were carried out with Amber-12[34] using ff12sb force field. Complexes were centered in a solvent box with boundaries located at 15 Å distance from the outermost solute atoms in each direction. Periodic boundary conditions were applied. The boxes were filled with water molecules with the TIP3P water model[35] and counterions (Na^+ and Cl^-) were added. For long-range electrostatics, Particle Mesh Ewald was used with cutoff of 14 Å. The potential energy of the system was initially minimized. The position restraints of the heavy atoms in the protein were released in 2 steps: (1) minimization with 2.4 kcal/(mol $Å^2$) position restraints on all the heavy atoms in the protein; (2) minimization performed without position restraints. Afterwards, the systems were simulated for 100 ps steps with gradual temperature increase from 0 to 50 K with restraints of 2.4 kcal/(mol $Å^2$) on all heavy atoms, from 50 to 200 K with restrains of 2.4 kcal/(mol $Å^2$) only on the backbone atoms, and from 200 to 300 K with restraints on the backbone atoms with force of 0.24 kcal/(mol $Å^2$). Finally, the protein molecules were simulated at a constant temperature (300 K) by using the Berendsen thermostat at default settings in the NPT ensemble for 10 ns in the production run. The analysis of the results was done with visual molecular dynamics (VMD) and PyMol (http://www.pymol.org/).

RESULTS

Recombinant TCRs 2D1 and B7 recognize plasmid-encoded peptides. The HLA-A3-PLP(45-53)- and HLA-A2-TAX(11-19)-specific TCRs 2D1[19] and B7[25] were functionally expressed in the murine T hybridoma cell line $58\alpha^-\beta^-$, which lacks endogenous TCR chains.[23] Both transfectants were additionally transfected with human CD8αβ chains.[20] Here we supertransfected them with the plasmid pcDNA-NFAT-sGFP,[22] which induces sGFP after TCR activation via the nuclear factor of activated T cells (NFAT). These engineered cells are termed 58-2D1-CD8-sGFP and 58-B7-CD8-sGFP. As antigen-presenting cells (APC), we used COS-7 cells that were transfected with HLA-A2 or HLA-A3.[22]

To validate functionality of the TCR transfectants and APCs, we cocultured 58-2D1-CD8-sGFP or 58-B7-CD8-sGFP cells with COS-7-A3 or COS-7-A2 cells, which were preincubated with appropriate synthetic peptides. As readout for TCR activation, we observed NFAT-sGFP induction by fluorescence microscopy and secretion of IL-2 into the supernatant (figure e-1 at Neurology.org/nn). We only observed TCR activation of 2D1 when HLA-A3 and PLP(45-53) were used. Vice versa, TCR B7 was only activated by HLA-A2 and TAX(11-19). Next, we confirmed that 58-2D1-CD8-sGFP and 58-B7-CD8-sGFP cells also recognized peptides that were encoded by plasmids and expressed in the cytosol of COS-7-A2 and COS-7-A3 cells (figure e-2). The activation pattern was identical to the pattern found with synthetic peptides (figure e-1), providing evidence that the TCR-transfected T hybridoma cells are functional, i.e., express the TCRs 2D1 or B7, both in conjunction with sGFP under the control of NFAT; that the

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COS-7 cells express functional HLA-A2 and -A3 molecules; and that plasmid-encoded peptides are efficiently produced and presented by COS-7 cells. Of note, TCR 2D1 recognized its specific peptide PLP(45-53) only in the context of its genuine restriction element HLA-A3, but not in the context of HLA-A2.

Mimotopes and natural peptides recognized by TCR 2D1 in the context of HLA-A2. To identify mimotopes recognized by TCR 2D1, we transiently transfected a PECP library into COS-7-A2 cells. The library (termed $8X^{8L}$) contained 7 randomized amino acids (X) at positions 1–7 and the HLA-A2 anchor amino acid leucine at position 8 (table). After 48 hours, we superposed the adherent COS-7-A2 cells by 58-2D1-CD8-sGFP cells. Thus, if one of the APCs presents a library peptide that is recognized by the TCR 2D1, the 58-2D1-CD8-sGFP cells that are in contact with this APC will fluoresce green and may be detected under a fluorescence microscope (figure e-3). We isolated the green 58-2D1-CD8-sGFP cells together with the subjacent COS-7-A2 and after subcloning,[22] we identified the plasmid-encoded mimotopes. Using the $8X^{8L}$ library, we identified 3 mimotopes (termed mimo-1 to mimo-3) (table).

From the mimotope sequences, we generated a search matrix for database searches (table). To this end, we assumed relative contributions of each amino acid for each position based on the sequences of the mimotopes and on known HLA-A2 binding motifs.[26] The search matrix was used to screen databases of human and murine proteomes that were confirmed at protein level. We tested 34 candidate peptides by transfecting COS-7-A2 cells with plasmids encoding the peptides and recording activation of 58-2D1-CD8-sGFP cells, and identified 4 peptides that were identical in human and mice that activated 58-2D1-CD8-sGFP cells, namely GPCPD1 (15-22), DMXL2(813-820), EML5(997-1004), and NCAN(257-264) (table). Further, we found 5 peptides from mice but not humans and 3 peptides from humans but not mice.

Recognition of HLA-A2:peptide complexes by TCR 2D1. Because plasmid-encoded peptides are initially produced with a N-terminal methionine (Met; M) and the efficiency of its cleavage may vary,[36] we compared peptides with or without their N-terminal Met. To this end, we incubated COS-7-A2 cells with octameric synthetic peptides and tested for recognition by 58-2D1-CD8-sGFP cells (figure 1, A–D). Only GPCPD1(15-22) induced considerable activation of 2D1 as observed by fluorescence microscopy (figure 1, left) and IL-2 production (figure 1, right). The signals of the other peptides were weak. However, when we tested synthetic peptides extended with an N-terminal Met, we observed considerable activation of 2D1 by all peptides (figure 1, E–H). Only GPCPD1(14-22) was activating 2D1 as a nonameric peptide with the natural Leu residue at its N-terminus. The other 3 peptides, which have Lys, His, and Glu residues at their N-termini, did not activate 2D1 (figure 1, I–L). This comparison eliminated 3 candidate peptides, leaving GPCPD1 as sole antigen. Of note, Leu is homologous to Met, whereas Lys, His, and Glu are distinct. Transfection of the relevant domain of the parent protein GPCPD1(1-118) into COS-7-A2 cells and presentation to 58-2D1-CD8-sGFP cells did not trigger activation of TCR 2D1 (figure 1M) although we could detect protein production in the cytosol of COS-7-A2 cells by Western blotting (data not shown). This indicates that COS-7 cells are unable to process the full-length protein appropriately.

As impaired TCR activation may be due to abolished peptide:HLA binding or to diminished recognition by the TCR, we compared the capacities of octamer and nonamer peptides for binding and stabilizing HLA-A2 by an in vitro refolding assay. We refolded the purified HLA-A2 heavy chain and β2m in the presence of M-GPCPD1(15-22) (figure 2A, lanes 2 and 4) and GPCPD1(15-22) (figure 2A, lanes 3 and 5). Under reducing conditions (lanes 2 and 3), the unfolded HLA-A2 heavy chains migrated at 39 kDa whereas refolded heavy chains migrated at 36 kDa under nonreducing conditions (lanes 4 and 5). The refolding yield was significantly higher in the presence of M-GPCPD1(15-22) as seen by the higher intensity of the 36 kDa band in lane 4 as compared to GPCPD1(15-22) in lane 5. We then biotinylated the refolded trimeric complexes at their BirA sites, immobilized them on streptavidin-coated microtiter plates, and used them to activate TCR 2D1. Although octameric GPCPD1(15-22) induced some HLA-A2 refolding, only the nonameric M-GPCPD1(15-22) was able to activate 58-2D1-CD8-sGFP cells as detected by fluorescence microscopy and secreted IL-2 in the supernatant (figure 2, B and C). This shows that TCR 2D1 requires a structure induced by the nonameric peptide for activation.

Since 2D1 cells express 1 β-chain and 2 α-chains (Vα2Jα21 and Vα7Jα11), we compared 2D1 with $58\alpha^-\beta^-$ cells that were transfected with the β-chain and either of the α-chains[20] for recognition of HLA-A3-PLP(45-53) and HLA-A2-GPCPD1(15-22). We found that the dual-α 2D1 cells showed identical pattern to the Vβ15.1Jβ2.7-Vα2Jα21 transfectants, whereas Vβ15.1Jβ2.7-Vα7Jα11 transfectants recognized neither of the complexes (figure 3, A–C). This shows that the Vβ15.1Jβ2.7-Vα2Jα21 TCR is cross-reactive to both HLA:peptide complexes.

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Table Peptides and antigen search matrix

Position	1	2	3	4	5	6	7	8	9
PLP(45-53)	K	L	I	E	T	Y	F	S	K
8X^{8L}		X	X	X	X	X	X	X	L
Mimo-1		L	I	G	E	V	F	V	L
Mimo-2		L	V	G	E	V	W	G	L
Mimo-3		L	L	G	E	V	F	E	L
GPCPD1(14-22)	I	L	P	G	E	V	F	A	I
DMXL2(812-820)	k	L	I	G	E	V	F	N	I
NCAN(256-264)	e	L	G	G	E	V	F	Y	V
EML5(996-1004)	h	M	E	G	E	V	W	G	L
Search matrix									
A		—	5	—	—	—	—	5	—
C		—	—	—	—	—	—	5	—
D		—	—	—	10	—	—	5	—
E		—	—	—	80	—	—	5	—
F		—	—	—	—	—	40	5	—
G		—	5	100	—	—	—	5	—
H		—	—	—	—	—	5	5	—
I		—	30	—	—	—	—	5	10
K		—	—	—	—	—	—	5	—
L		80	30	—	—	—	—	5	70
M		20	—	—	—	—	—	5	10
N		—	—	—	—	—	—	5	—
P		—	—	—	—	—	—	5	—
Q		—	—	—	10	—	—	5	—
R		—	—	—	—	—	—	5	—
S		—	—	—	—	—	—	5	—
T		—	—	—	—	—	—	5	—
V		—	30	—	—	100	—	5	10
W		—	—	—	—	—	40	5	—
Y		—	—	—	—	—	15	5	—
Sum		100	100	100	100	100	100	100	100

Abbreviations: DMXL2 = Dmx-like-2; GPCPD1 = glycerolphosphatidylcholine phosphodiesterase 1; PLP = proteolipid protein; EML5 = echinoderm microtubule associated protein-like 5; NCAN = Neurocan.

The uppermost line lists the position of the amino acids, line 2 shows the sequence of the human leukocyte antigen (HLA)–A3-presented peptide PLP(45-53), line 3 the plasmid-encoded combinatorial peptide library 8X^{8L}, lines 4–6 the mimotopes identified for HLA-A2 recognition by T-cell receptor 2D1, and lines 7 to 10 the abbreviated names and sequences of 4 peptides that are identical in mice and humans. All amino acids are given in single letter code. We normalized the numbering to nonameric peptides. The peptides GPCPD1(14-22), DMXL2(812-820), NCAN(256-264), and EML5(996-1004) were tested as 9mers and as 8mers with the first amino acid missing. Therefore the first amino acids are printed in lowercase. In the combinatorial peptide library 8X^{8L} X denotes a random amino acid encoded by the nucleotides NNK. Lines 11–30 show the search matrix for database searches. Based on the mimotope sequences and HLA-A2 binding motifs, we attributed relative contributions of each possible amino acid for each position similar to a described method.[40] We list all 20 amino acids in alphabetical order in the first column. For each amino acid and each position, we denote their relative contribution to the search matrix. Their contribution to each position is summed up to 100% (line 32). For example, we assumed exclusive contribution of G and V in positions 4 and 6, respectively, aliphatic amino acids with strong biases on L at the binding positions 2 and 9, and complete random distributions at position 8, because of the very different chemical properties of the 3 amino acids V, G, and E found here in the 3 mimotopes.

Molecular modeling. We simulated the structures of the octameric and nonameric complexes in silico. Starting with the known structure of the HLA-A2-flu(58-66) and -Tax8 complexes,[31,32] we replaced the peptides by GPCPD1(15-22), M-GPCPD1 (15-22), and GPCPD1(14-22). From a molecular dynamics simulation, it is evident that there are significant differences in the positions of the polypeptide backbones and in the positions of the amino acid side chains of the octameric and nonameric peptides (figure 4, A and B), in particular in the middle of the peptides, which are supposed to interact with the TCR. For example, F20 is oriented towards the HLA floor in GPCPD1(14-22) and M-GPCPD1(15-22), but reaches towards the TCR in the octameric GPCPD1 (15-22). Another difference regards the first amino acids of the nonameric peptides, Met or Leu, respectively, which form hydrogen bonds with 3 different Tyr residues of HLA-A2 (Y171, Y159, and Y7) (figure 4C), whereas there are no analogous interactions in the octamer (figure 4D). We compared the flexibility in terms of root mean square deviation (RMSD) of the peptide atoms by using VMD.[37] Thus, the octamer GPCPD1(15-22) ($RMSD^{GPCPD1}_{[15-22]}$ = 2.130 Å) was significantly more flexible as the nonamers ($RMSD^{M\text{-}GPCPD1[15\text{-}22]}$ = 1.272 Å, $RMSD^{GPCPD1[14\text{-}22]}$ = 1.474 Å). This indicates less stable binding and might explain its weaker potential to stabilize the complex (figure 2, B and C).

DISCUSSION

We identified antigenic peptides that are presented by the protective allele HLA-A2 to TCR 2D1, which was originally generated against the myelin autoantigen peptide PLP(45-53) in the context of HLA-A3. Our unbiased antigen-identification technology revealed several T-cell antigens on an allogeneic MHC background, which allowed for molecular characterization of HLA:peptide complexes. The identification of autologous peptides recognized by the HLA-A3 restricted TCR 2D1 in context of HLA-A2 underlines the advantages of PECP libraries.[22] First, they are independent of any—often extremely rare—primary cells; second, they are independent of any antigen processing mechanisms; third, they do not require high affinities or avidities between MHC:peptide complexes and TCR, because APCs and T cells are held in contact by gravity; and fourth, they are unbiased, i.e., no hypothetical candidate antigens are required. This technology therefore is a promising tool for identifying mimotopes and based on that, candidate antigens of CD8+ T cells that are disease relevant in autoimmunity, tumors, and infections.

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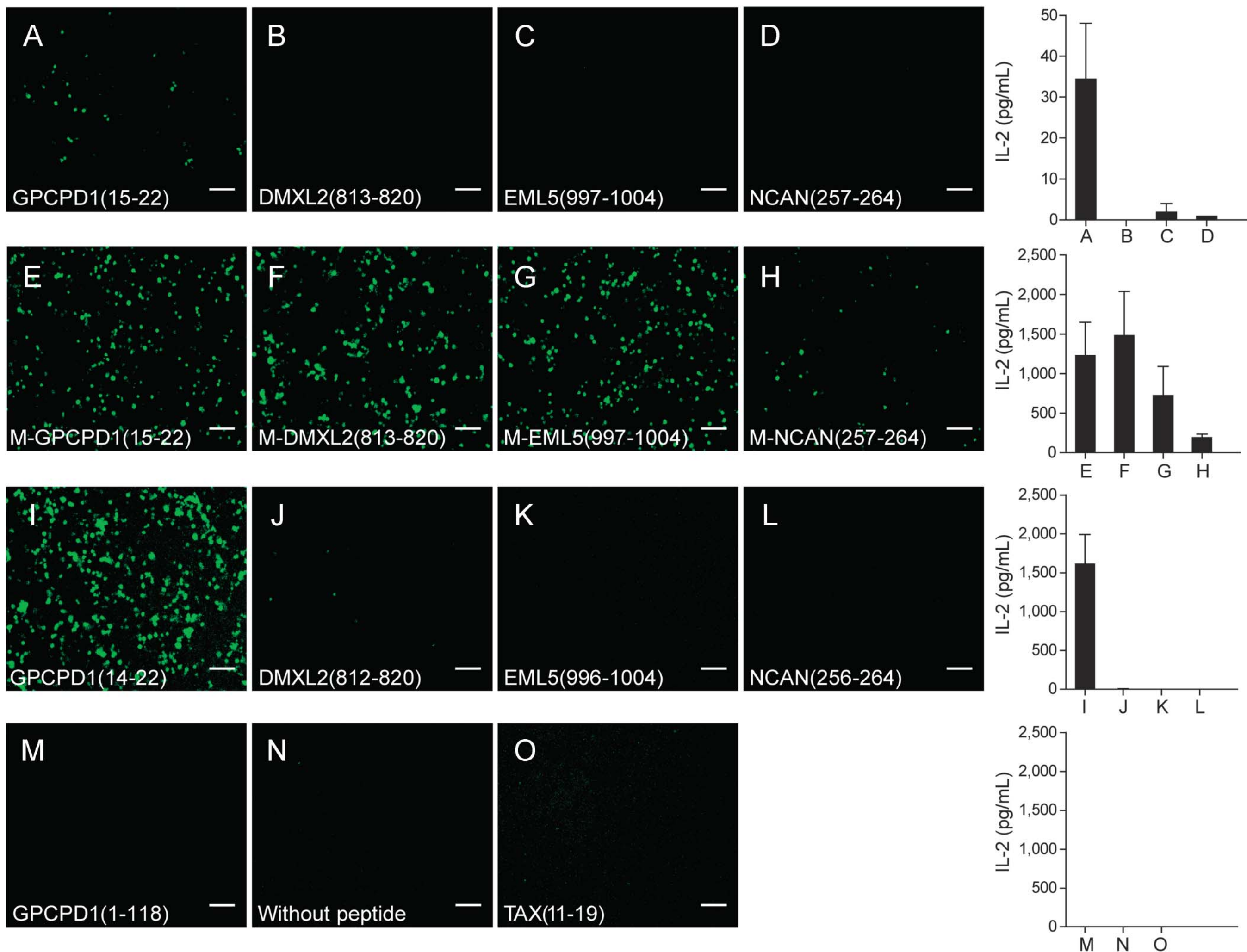
Figure 1 T-cell receptor 2D1 recognizes human leukocyte antigen (HLA)-A2-restricted octameric and nonameric synthetic peptides



Left: Fluorescence microscope images from coculture of 58$\alpha^-\beta^-$ T hybridoma cells expressing TCR 2D1, human CD8$\alpha\beta$ molecules, and sGFP under the control of nuclear factor of activated T cells (58-2D1-CD8-sGFP) and COS-7 cells expressing HLA-A*02:01(COS-7-A2) cells preincubated with synthetic peptides. Green cells indicate activation of 58-2D1-CD8-sGFP cells. The names of the peptides are given as inserts. Scale bars: 100 μm. Right: Secreted interleukin (IL)-2 as measured by ELISA of the coculture's supernatants. In the top row (A–D), octameric peptides were used. Only glycerolphosphatidylcholine phosphodiesterase 1 (GPCPD1) (15-22) weakly activated 58-2D1-CD8-sGFP cells. In the second row (E–H), the same octameric peptides were used but all carried an additional methionine residue at their N-terminus. All of them strongly activated 58-2D1-CD8-sGFP cells. In the third row (I–L), the same octameric peptides were used but all carried their original amino acid residue at their N-terminus. Only GPCPD1(14-22) activated 58-2D1-CD8-sGFP cells. Lowest row: (M) The entire domain of GPCPD1(1-118) that harbors the antigenic peptide GPCPD1(14-22) did not activate 58-2D1-CD8-sGFP cells when transfected into COS-7-A2 cells. When no peptide (N) or the irrelevant peptide T-lymphotrophic virus-2 protein (TAX) (11-19) (O) was added, 58-2D1-CD8-sGFP cells were not activated. Error bars represent standard deviation of the mean. DMXL2 = Dmx-like-2; EML5 = echinoderm microtubule associated protein-like 5; NCAN = Neurocan.

PECP libraries reveal mimotopes, and from these several candidate peptides may be delineated based on their presumed biological relevance. Here we were focusing on candidate peptides that are expressed both in humans and mice. Biochemical follow-up experiments, such as analysis of the N-terminal processing patterns, further narrowed the selection of biologically relevant candidate peptides. Here, 3 of 4 candidate peptides did not stimulate 2D1 as octamers and as nonamers when the natural N-terminal amino acid was added. Only (GPCPD1[14-22]) activated 2D1 as nonamer when the naturally occurring Leu replaced Met, which is introduced by inefficient cleavage of the PECP library. Even octameric GPCPD1(15-22) activated 2D1, though to a lesser extent. By molecular modeling, we found that both nonameric peptides adopt similar conformations whereas the octamer backbone was distorted throughout the entire length of the peptide. The first amino acids of both nonameric peptides mounted hydrogen bonds to 3 different tyrosine residues of HLA-A2, influencing the structure and flexibility and consequently also the function of HLA-A2. This is presumably why the octamer

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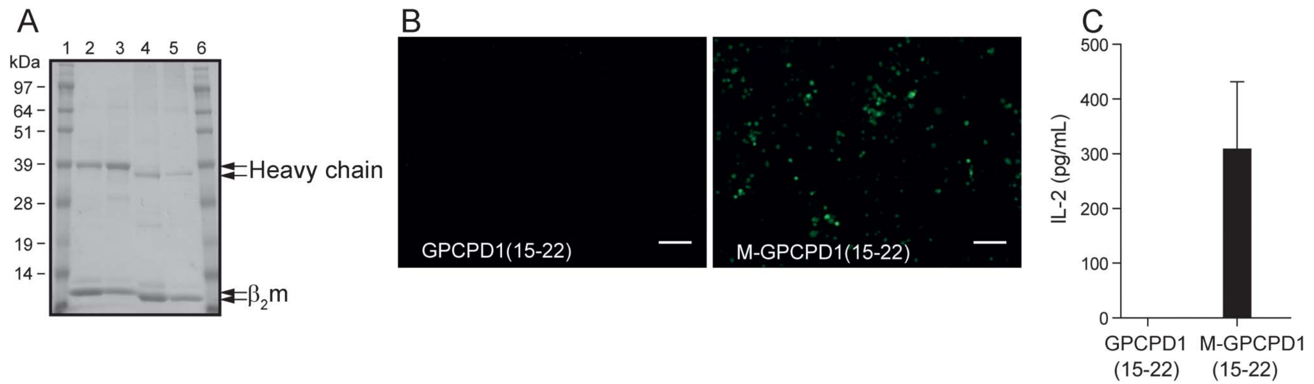
Figure 2 In vitro refolded human leukocyte antigen (HLA)-A2:peptide complexes activate TZR 2D1



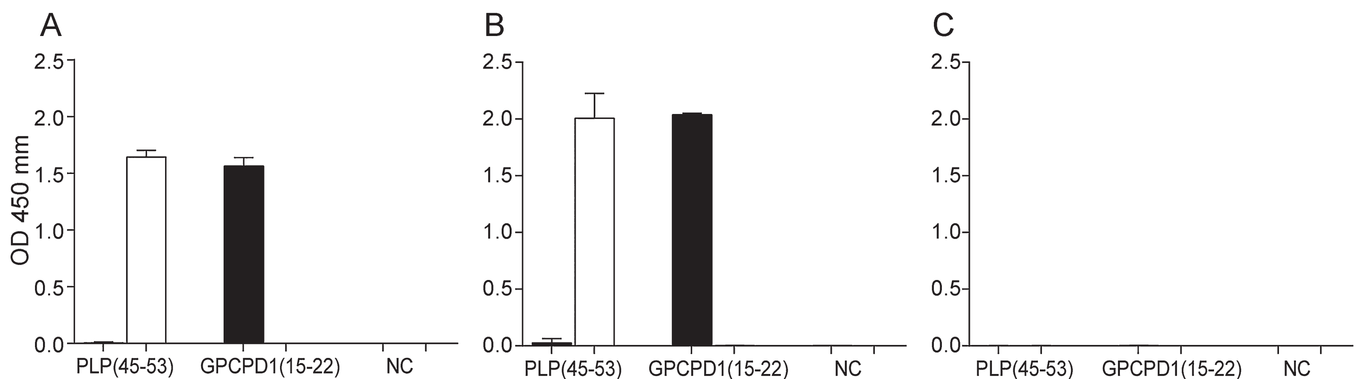
(A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of HLA-A*02:01 (HLA-A2) refolded from recombinant heavy chain and β2m in the presence of either the octameric glycerolphosphatidylcholine phosphodiesterase 1 (GPCPD1) (15-22) or the nonameric M-GPCPD1(15-22) under reducing and non-reducing conditions. Lanes 1 and 6, protein standard. Molecular masses are given in kDa at the left; lane 2, HLA-A2, β2m, and M-GPCPD1(15-22) under reducing conditions; lane 3, HLA-A2, β2m, and GPCPD1(15-22) under reducing conditions; lane 4, HLA-A2, β2m, and M-GPCPD1(15-22) under nonreducing conditions; lane 5, HLA-A2, β2m, and GPCPD1(15-22) under nonreducing conditions. (B) Activation of $58\alpha^-\beta^-$ T hybridoma cells expressing TCR 2D1, human CD8αβ molecules, and sGFP under the control of nuclear factor of activated T cells (58-2D1-CD8-sGFP) cells by in vitro refolded trimeric HLA-A2:β2m:peptide complexes as measured by fluorescence microscopy. Left panel: 58-2D1-CD8-sGFP cells were incubated with refolded and biotinylated HLA-A2, β2m, and GPCPD1(15-22). Right panel: As left panel, but with M-GPCPD1(15-22). Scale bars: 100 μm. (C) IL-2 concentration measured in the supernatant from the experiments shown in (B). Error bars represent SD of the mean.

activates TCR 2D1 only weakly and catalyzes refolding to a lesser extent.

Comparison of our model of the allogeneic GPCPD1(14-22):HLA-A2 complex to the X-ray structure of the parent PLP(45-53):HLA-A3 complex[38] reveals similar surfaces. The amino acids of both HLA-A2 and HLA-A3 that face the TCR are identical. L15 of GPCPD1 and L46 of PLP occupy the canonical pocket B, and the C-terminal amino acids are bound to pocket F. Positions 3 and 8 of the HLA-A2 binding mimotopes and candidate peptides (table) contain diverse amino acids and are presumably irrelevant for HLA binding and TCR recognition. Significant differences are only observed at positions 4 to 6, where PLP(45-53) reads Glu-Thr-Tyr, but all mimotopes and candidate peptides read Gly-Glu-Val. However, it is unknown how these different structures are interacting with TCR 2D1.

Our current results provide proof of principle that our technique[22] allows unbiased identification of a cross-reactive peptide recognized by a human myelin-reactive TCR that was originally isolated from a PLP-specific HLA-A3-restricted CD8+ T-cell clone obtained from a patient with MS. We are, however, fully aware that our demonstration of such

Figure 3 The T-cell receptor (TCR) Vα2Jα21-chain determines cross-reactivity of 2D1 cells



Activation of TCR-transfected T hybridoma cells by human leukocyte antigen (HLA)-transfected COS-7 cells and plasmid-encoded peptides. Adherent COS-7 cells expressing HLA-A*02:01 (COS-7-A2) (shaded bars) or COS-7 cells expressing HLA-A*03:01 (COS-7-A3) (white bars) were transfected with plasmids coding for proteolipid protein (PLP) (45-53), glycerolphosphatidylcholine phosphodiesterase 1 (GPCPD1) (15-22), or empty plasmid (NC). Transfected COS-7 cells were superposed with 58α-β-cells that were transfected with CD8, the TCR Vβ15.1Jβ2.7 β-chain, and (A) both α-chains (Vα2Jα21 and Vα7Jα11), (B) the Vα2Jα21-chain alone, or (C) the Vα7Jα11-chain alone. T-cell activation was analyzed by measurement of secreted IL-2 by ELISA. Error bars represent SD of the mean.

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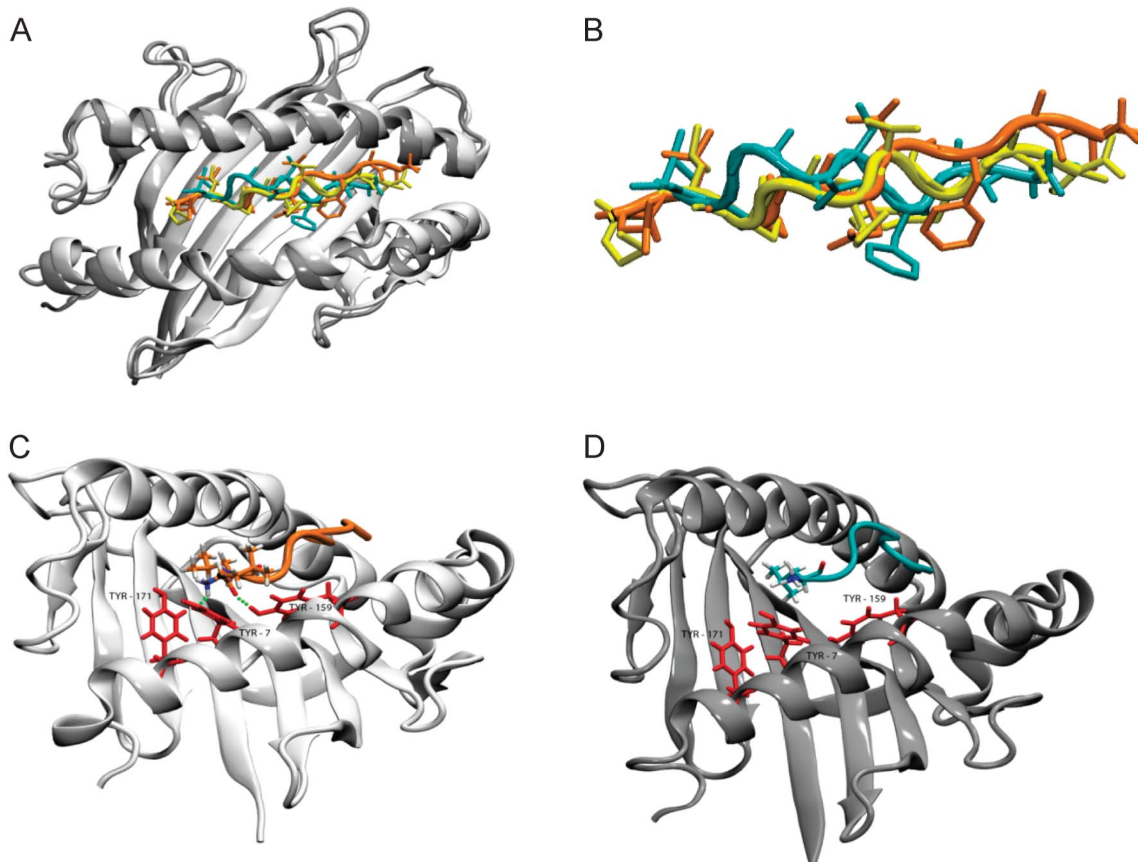
Figure 4 **Molecular models of antigenic human leukocyte antigen (HLA)-A2:peptide complexes by molecular dynamics simulations**



(A) Aligned structures of the peptides glycerolphosphatidylcholine phosphodiesterase 1 (GPCPD1) (14-22):LLPGEVFAI (orange), M-GPCPD1(15-22): MLPGEVFAI (yellow), and GPCPD1(15-22):LPGEVFAI (cyan) bound to HLA-A*02:01 (HLA-A2) after 10 ns molecular dynamics run. HLA-A2 with bound nonamers GPCPD1(14-22) and M-GPCPD1(15-22) is shown in light gray and HLA-A2 with bound octamer GPCPD1(15-22) is shown in dark gray. The HLA: peptide complexes are shown from the top, i.e., from the position of the TCR. (B) The 3 peptides (see A) shown for clarity in greater magnification without the HLA molecules. The diverse courses of the polypeptide backbones and the different positions of the amino acid side chains are evident. These differences are particularly pronounced in the region around F20, which is facing towards the HLA floor for the nonamers but faces upward in the octamer. (C) View from the N-terminus of HLA-A2 with bound nonameric GPCPD1(14-22) (orange). The residues Y171, Y159, and Y7 have stabilizing effect on the bound nonamer and are shown in red. This tyrosine triad interacts with the N-terminal leucine of the bound peptide by forming hydrogen bonds that anchor the peptides tightly. (D) View from the N-terminus of the HLA-A2 with bound octameric GPCPD1(15-22) (cyan). The bound octamer does not form stabilizing hydrogen bonds with the tyrosine residues, thus appearing as more flexible during the molecular dynamics simulations.

molecular mimicry between 2 different HLA class I molecules that present different peptides does not prove relevance in vivo. Thus, it is not known whether GPCPD1(14-22) is tolerogenic in the triple transgenic mice. Addressing this question requires additional in vivo and in vitro studies, notably of thymic processing and presentation of the parent full-length GPCPD1 protein in medullar thymic epithelial cells.[39] In addition, we do not know whether the peptide is naturally processed and presented in peripheral immune organs or the CNS. Clearly, and in contrast to the findings in EAE, TCR 2D1-expressing T cells were not deleted in the HLA-A2+/HLA-A3+ patient from whom the T-cell clone was originally isolated. Nevertheless, our findings may serve as a paradigm of a CD8+ T-cell mimicry interaction between a well-characterized antimyelin TCR with a class I HLA molecule that confers protection from MS. In the long run, our antigen search technology may be applied to TCR from CD8+ T cells isolated from MS lesions, which may provide insight into MS pathogenesis.

AUTHOR CONTRIBUTIONS

G.R. and A.G.N. performed experiments and analyzed and interpreted data. A.P., M.K., and I.A. performed molecular modeling and molecular dynamics experiments and analyzed and interpreted these data. K.S. contributed to antigen search experiments. S.P. performed database analyses. M.A.F. and K.E.A. contributed samples. R.H. contributed to the design of the study, interpretation of the results, and writing the manuscript. K.D. conceived and coordinated the study and wrote most of the manuscript. All authors contributed to writing, reviewed the results, and approved the final version of the manuscript.

ACKNOWLEDGMENT

The authors thank Joachim Malotka and Ingrid Eiglmeier for technical assistance, Kerstin Berer, Jessica Bruder, and Simone Brändle for discussion, and Edgar Meinl and Gurumoorthy Krishnamoorthy for comments on the manuscript.

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STUDY FUNDING

Supported by grants from the Deutsche Forschungsgemeinschaft (CRC-TR-128-A5 and 128-B8, Center for Integrated Protein Science Munich [CIPSM], Munich Cluster for Systems Neurology [EXC 1010 SyNergy]). A.G.N. was supported by the Studienstiftung des Deutschen Volkes, M.A.F. by the Deutsche Forschungsgemeinschaft Emmy Noether Programme (FR1720/3-1), and K.E.A. by the MRC UK.

DISCLOSURE

G. Rühl reports no disclosures. A.G. Niedl received research support from Studienstiftung des Deutschen Volkes. A. Patronov, K. Siewert, S. Pinkert, and M. Kalemanov report no disclosures. M.A. Friese received research support from German Research Foundation, German Ministry of Education and Research. K.E. Attfield and I. Antes report no disclosures. R. Hohlfeld served on the advisory board for Novartis, Biogen Idec, Bayer-Schering, Merck-Serono, Sanofi-Aventis, Teva, CSL Behring, Medday, and Acelion; received travel funding from Novartis, Biogen-Idec, Bayer-Schering, Merck-Serono, Sanofi-Aventis, Teva, and Genzyme; is/was on the editorial board for *Neurology®*, *Brain*, *Clinical and Experimental Immunology*, *Deutsche Medizinische Wochenschrift*, *Expert Opinion on Biological Therapy*, *Journal of Neuroimmunology*, *Multiple Sclerosis*, *Nervenarzt*, *Practical Neurology*, *Seminars in Immunopathology*, and *Therapeutic Advances in Neurological Disorders*; consulted for Novartis, Biogen Idec, Bayer-Schering, Merck-Serono, Sanofi-Aventis, Teva, Genzyme, Medday, and Actelion; and received research support from Novartis, Biogen-Idec, Bayer-Schering, and Teva. K. Dornmair received research support from Deutsche Forschungsgemeinschaft. Go to Neurology.org/nn for full disclosure forms.

Received January 20, 2016. Accepted in final form April 1, 2016.

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Neurol Neuroimmunol Neuroinflamm 2016;3;
DOI 10.1212/NXI.0000000000000241

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