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Ärztlicher Direktor: Prof. Dr. Johannes Dichgans

Experimentelle neuroimmungenetische Untersuchungen zur Autoaggression und Therapie der Multiplen Sklerose

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Dr. Dr. med. Robert Weißert
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Inhaltsverzeichnis

Abkürzungen

ACI Ratte	AxC 9935 Irish Ratte
APL	Altered peptide ligand
APZ	Antigen präsentierende Zelle
BN Ratte	Brown Norway Ratte
COP Ratte	Copenhagen 2331 Ratte
DA Ratte	Dark Agouti Ratte
EAE	Experimentelle autoimmune Enzephalomyelitis
ELISPOT	Enzyme-linked immunospot Assay
gp	Guinea pig, Meerschweinchen
H-2	MHC der Mäuse
HLA	Human leukocyte antigens, humanes Leukozyten Antigen
IFN	Interferon
IL	Interleukin
LEW Ratte	Lewis Ratte
MBP	Myelin-basisches Protein
MHC	Major histocompatibility complex, Transplantationsantigene
MOG	Myelin-Oligodendrozyten-Glykoprotein
MS	Multiple Sklerose
PLP	Proteolipid Protein
RT1	MHC der Ratte
TZR	T Zellrezeptor
TNF	Tumor Nekrose Faktor
WP Ratte	Wistar Prague Ratte
ZNS	Zentrales Nervensystem

EINFÜHRUNG IN DIE THEMATIK

Multiple Sklerose

Die Multiple Sklerose (MS) ist eine entzündliche mit Entmarkung des Myelins und Axonverlust einhergehende Erkrankung des zentralen Nervensystems (ZNS) (Noseworthy et al., 2000; Steinman 2001). Mit hoher Wahrscheinlichkeit liegt der Erkrankung eine Autoimmunpathogenese zu Grunde, bei der autoreaktive, ZNS-antigenspezifische T Zellen aktiviert werden, die weitere Immunkaskaden auslösen, welche zur Gewebsschädigung führen. Die Läsionen im ZNS treten multifokal auf und sind zu Beginn der Erkrankung vornehmlich im Marklager lokalisiert (Lassmann et al., 1994; Raine 1994). Im Verlaufe der Erkrankung kann es zu einer Beteiligung des gesamten ZNS kommen. Man unterscheidet unterschiedliche klinische Verläufe. So gibt es schubweise Verläufe sowie primär- und sekundär chronische Verläufe (Noseworthy et al., 2000). Schubweise Verläufe gehen in einem großen Prozentsatz in sekundär chronische Verläufe über. Die Diagnose wird anhand verschiedener klinischer Kriterien gestellt, wobei das Auftreten von Krankheitssymptomen, kernspintomographisch sichtbare Läsionen, verzögerte visuell evozierte Potentiale und spezifische Normabweichungen des Liquor cerebrospinalis gewertet werden (McDonald et al., 2001). Zur Suszeptibilität für die Erkrankung tragen genetische Prädispositionsfaktoren, welche vor allem mittels Zwillingsstudien und Studien an adoptierten Kindern nachgewiesen wurden, bei (Ebers et al., 1986; 1995; Willer and Ebers, 2000; Oksenberg et al., 2001). Zudem kommt Umweltfaktoren eine Rolle bei der Entstehung der MS zu (Willer and Ebers, 2000). Die Erkrankung ist mit hoher Wahrscheinlichkeit pathogenetisch heterogen. Derzeit wird diese Annahme vor allem durch histopathologische Untersuchungen von MS Läsionen gestützt (Lucchinetti et al., 2000).

Hauptsuszeptibilitätslokus - MHC

Die Gene, welche die Transplantationsantigene oder den Major Histocompatibility Complex (MHC), beim Menschen Human Leukocyte Antigens (HLA) kodieren, befinden sich auf dem Chromosom 6 und umfassen etwa 3,6 Mb. Der MHC des Menschen enthält ungefähr 120 exprimierte Gene (Bild 1) (The MHC sequencing consortium, 1999; Günther and Walter, 2001). Ein Teil dieser Gene kodiert Moleküle, welche T Zellen kurze Peptide präsentieren. Diese Gene und ihre Produkte sind sehr polymorph und werden häufig gemeinsam in Clustern vererbt (Klein and Sato, 2000).

Eine bestimmte allelische Spezifität solcher gemeinsam vererbter Gene wird Haplotypus genannt. Für den MHC des Menschen sind etwa 25 Krankheitsassoziationen bekannt (Klein and Sato, 2000). Der MHC beeinflusst die individuelle Suszeptibilität für Infektionskrankheiten. Dieses konnte am Beispiel der HIV Infektion, der Infektion mit Hepatitis B und C Viren und der Malaria gezeigt werden (Hill et al., 1991; Thursz et al., 1997; McDermott et al., 1999; Carrington et al., 1999; Thursz et al., 1999). Auch bei Tumoren findet sich eine MHC Assoziation (Bateman and Howell, 1999; Little and Stern, 1999). Auch die Suszeptibilität für viele Autoimmunerkrankungen ist mit verschiedenen HLA Haplotypen assoziiert. So konnten Assoziationen mit der rheumatoiden Arthritis (Andersson et al., 2000), dem Diabetes mellitus (Mein et al., 1998; Undlien 2001), der Myasthenia gravis (Moller et al., 1976), der Multiplen Sklerose (Dyment et al., 1997) und einer Reihe weiterer Autoimmunerkrankungen aufgezeigt werden (Klein and Sato, 2000).

Bei MS Patienten europäischer Abstammung findet sich eine Assoziation mit dem HLA Haplotypus DRB1*1501 DRB5*0101 DQB1*0602 (HLA-Dw2) (Hillert and Olerup, 1993). Dieser Haplotypus ist auch mit einem früheren Beginn der Erkrankung assoziiert (Masterman et al., 2000). Es ist bisher nicht geklärt, ob DRB1*1501 oder DQB1*0602 oder beide Gene Suszeptibilitätsallele darstellen, da diese bei Europäern gemeinsam vererbt werden. In China und Brasilien finden sich bei einigen Populationen rekombinante MHC Haplotypen, in welchen DQB1*0602 unabhängig von DRB1*1501 vererbt wird. In diesen Populationen ist die MS mit der allelischen Spezifität DQB1*0602 assoziiert (Serjeantson et al., 1992; Caballero et al., 1999). Die Ursache für die MHC Assoziation bei MS Patienten ist bisher nicht bekannt. Es wird angenommen, dass MHC Moleküle T Zellen Fragmente von Myelinbestandteilen präsentieren. T Zellen werden dadurch aktiviert und stoßen weitere Immunkaskaden an (Martin et al., 1992). Große Genomanalysen haben bisher keine weiteren Gene oder Genregionen sicher eingrenzen können, allerdings finden sich deutliche Hinweise darauf, dass eine Vielzahl von Genen an der Entstehung der MS beteiligt sind (The Transatlantic Multiple Sclerosis Genetics Cooperative, 2001). Mittels verbesserter Methoden und einem größeren Patientenmaterial wird es in den nächsten Jahren möglich sein, solche Gene zu kartieren.

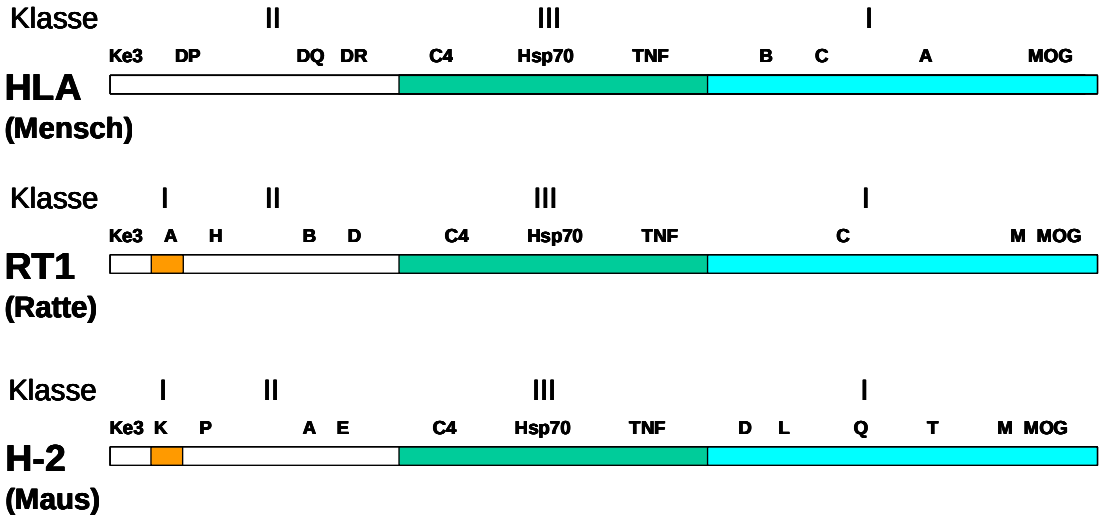

Bild 1. Aufbau des MHC beim Menschen (HLA auf Chromosom 6), der Ratte (RT1 auf Chromosom 20) und der Maus (H-2 auf Chromosom 17). Graphik adaptiert nach Günther (Günther, 1996; Günther and Walter, 2001).

Therapie der MS

Erst in den letzten Jahren ist es möglich geworden, neue und verbesserte Therapien für MS Patienten anzubieten (Noseworthy et al., 2000). Dazu gehören die verschiedenen Herstellungen von Beta-Interferon und Copaxone (COP-1), einem Copolymer aus vier Aminosäuren (Glutaminsäure, Alanin, Lysin, Tyrosin). Diese Substanzen reduzieren bei MS Patienten Schübe, ZNS Läsionen und die Progression. Daneben werden Kortikoide in der Akuttherapie und bei schnell progredienten Verläufen, vor allem bei einem drohenden Verlust der Gehfähigkeit, Chemotherapeutika eingesetzt. Sowohl die immunmodulatorischen als auch die immunsuppressiven Substanzen beeinflussen die entzündliche Phase der Erkrankung. Die schleichende Progression der neurologischen Ausfallserscheinungen, welche vornehmlich in der sekundär chronisch progredienten Erkrankungsform, aber auch bei der primär chronisch progredienten MS auftritt, wird durch diese Maßnahmen nur gering beeinflusst. In diesen Stadien der Erkrankung bestimmt vor allem der Axon- und Neuronenverlust die Progression (Trapp et al., 1998). Für diese Erkrankungsformen gibt es bisher keine etablierten medikamentösen Therapien, welche die Krankheitsprogression maßgeblich beeinflussen. Die Therapie ist symptomorientiert und physikalische Maßnahmen (Rehabilitation) sind angezeigt. Bei der schubweisen MS bedingt die akute

Entzündung neben dem Untergang von Markscheiden auch einen frühen Verlust von Axonen und Nervenzellen (Coles et al., 1999). Dieser frühe Verlust kann durch immunmodulatorische Maßnahmen reduziert werden. Daher wird heute vertreten, dass die Behandlung der MS mit immunmodulatorischen Maßnahmen frühestmöglich beginnen sollte (Noseworthy et al., 2000).

Myelinscheide

Die Myelinscheide der Axone im ZNS wird von Oligodendrozyten gebildet (Baumann and Pham-Dinh, 2001). Sie stellt eine extrazelluläre Organelle der Oligodendrozyten dar und enthält die wesentlichen Antigene für den Autoimmunprozess bei der MS. Sie besteht aus Lipiden und verschiedenen Proteinen. Ein großer Anteil wird von den Strukturproteinen Myelin-basisches-Protein (MBP) und Proteolipid-Protein (PLP) gebildet. Auf der Außenseite und Innenseite der Myelinscheide befinden sich Moleküle, deren Funktion noch wenig bekannt sind. Ein solches Protein stellt das auf der Außenseite lokalisierte Myelin-Oligodendrozyten-Glykoprotein (MOG) dar (Bild 2). Dieses bildet nur etwa 0,01 % der Proteinfraktion der Myelinscheide (Bernard et al., 1997).

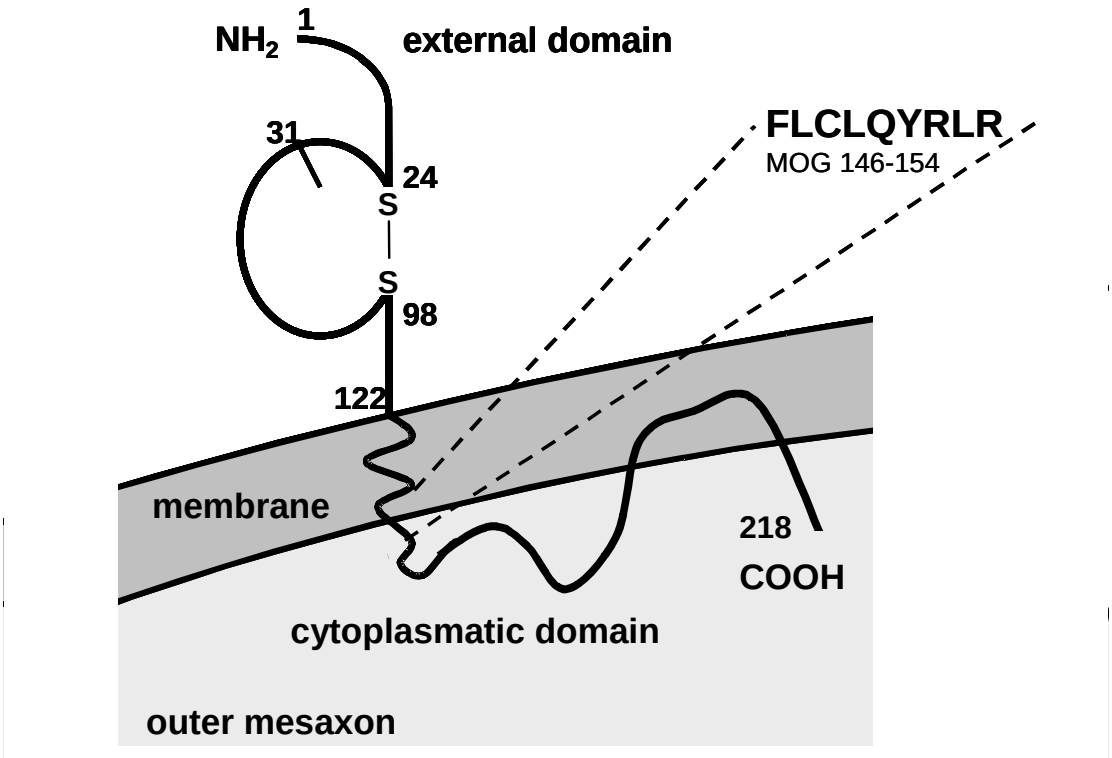

Bild 2. Myelin-Oligodendrozyten-Glykoprotein (MOG).

In der vorliegenden Arbeit werden die enzephalitogenen Eigenschaften von MOG im Rattenmodell und einem humanisierten Mausmodell der MS genauer analysiert.

Experimentelle autoimmune Enzephalomyelitis

Die experimentelle autoimmune Enzephalomyelitis (EAE) ist ein induziertes Tiermodell der MS, welches durch aktive und passive Immunisierung in Mäusen, Ratten, Affen und anderen Spezies ausgelöst werden kann (Wekerle et al., 1994; Gold et al., 2000). Bei der aktiven Immunisierung werden Myelinbestandteile zusammen mit einem Adjuvans (im Allgemeinen komplettes Freund's Adjuvans [besteht aus Mineralöl und Hitze-inaktivierten Bestandteilen von Mykobakterien] oder/und Pertussis Toxin) den Tieren intradermal oder subkutan verabreicht. Dadurch wird eine Immunreaktion mit Aktivierung von dendritischen Zellen ausgelöst, die eine adaptive Immunantwort der T- und B Zellen initiieren. Die dabei aktivierten enzephalitogenen T Zellen wandern in das ZNS und lösen dort weitere Immunkaskaden aus.

Bei der aktiven Immunisierung werden die Tiere in der Regel nach 10-15 Tagen krank. Bei der passiven Immunisierung werden den Tieren aktivierte T Zellen, die für gewisse Myelinbestandteile spezifisch sind, injiziert. Diese aktivierten T Zellen wandern in das ZNS und lösen dort Krankheitssymptome aus. Die passive Immunisierung führt in der Regel nach drei Tagen zu einer Erkrankung. Beide Verfahren der Krankheitsinduktion haben einen hohen Stellenwert, werden jedoch zur Untersuchung unterschiedlicher Fragestellungen angewendet.

Die EAE ist ein gutes Modell zu Untersuchung von pathogenetischen Prinzipien, welche der MS zugrunde liegen können (Wekerle et al., 1994). Durch die Verfügbarkeit von ingezüchteten Tierstämmen, kann die Modellerkrankung beliebig oft und unter den gleichen Bedingungen induziert werden und experimentelle Manipulationen vorgenommen werden. So ist dieses Modell auch gut dazu geeignet neue experimentelle Therapieverfahren zu erproben.

Die EAE wird durch MHC Gene und nicht-MHC Gene kontrolliert. So konnten bereits mehr als 20 Genorte eingegrenzt werden, die einen Einfluss auf die Suszeptibilität und den Verlauf der EAE haben (Butterfield et al., 2000). Einige dieser Loci sind

homolog zu Regionen, die beim Menschen die MS Suszeptibilität kontrollieren (Xu et al., 2001).
Man unterscheidet unterschiedliche klinische Verläufe bei der EAE. Im klassischen EAE Modell, der mit MBP immunisierten LEW Ratte, tritt ein selbstlimitierender monophasischer Verlauf auf. Darüber hinaus können mit anderen Antigenen und Tierstämmen schubförmige und chronische Verläufe induziert werden.

Kohärenz der Arbeiten

In der vorliegenden Arbeit wurde mit Hilfe der EAE der Einfluss von genetischen und von Umweltfaktoren auf die Krankheitspräzipitation (Suszeptibilität), den Verlauf der Erkrankung, Läsionsentstehung und immunologische Parameter untersucht. Als Beispiel für einen Umweltfaktor auf dem Boden einer genetischen Prädisposition wurde der Einfluss vom Austausch einer Aminosäure in einer enzephalitogenen Determinante des MBP, MBP 63-88, und der damit verbundenen Transition von einem Selbst- auf ein Fremdantigen untersucht (Publikation 1). Der Einfluss des Genoms wurde durch Variation von MHC und nicht-MHC Genen mittels einer Vielzahl ingezüchteter Rattenstämme untersucht (Tafel 1). Dabei wurde ein verbessertes Modell der MS in Ratten mittels aktiver Immunisierung mit MOG entwickelt (Publikation 3). In diesem Modell wurde eine Feinanalyse der T- und B-Zellenreaktivitäten in Bezug auf die Auslösbarkeit von Erkrankung erarbeitet (Publikation 4). In einem humanisierten Mausmodell, in welchem das DRB1*0401 Allel exprimiert wird, wurde die Enzephalitogenität von MOG untersucht (Publikation 5). Das T-Zellrepertoire gegenüber MOG von MS Patienten und Kontrollen wurde beschrieben und eine dominante Determinante gefunden (Publikation 7). Mittels kombinatorischer, randomisierter Peptidbibliotheken wurde ein rationaler therapeutischer Ansatz in der MOG-EAE der Ratte zur Verhinderung der Erkrankung entwickelt, bei welchem hoch affin an Suszeptibilitäts- assoziierte MHC Klasse II Moleküle bindende Peptide hergestellt wurden, die keine Sequenzhomologien zu Selbstpeptiden aufwiesen (Publikation 6). Die Immunisierung mit nackter DNS kann zur Protektion vor verschiedenen Infektionskrankheiten eingesetzt werden. Wir haben das Potential von nackter DNS, welche für autoantigene Sequenzen kodiert, Krankheit zu unterdrücken, untersucht (Publikation 2). Die Publikationen haben die Grundlage für weiterführende Arbeiten sowohl im Tiermodell als auch bei MS

Patienten gelegt und lassen hoffen, dabei die Basis für ein neues Therapieprinzip bei MS Patienten etabliert zu haben.

Stamm*		***Klasse I***	***Klasse II***		***Klasse III***	***Klasse I***
	Haplotyp	RT1.A	RT1.B	RT1.D		RT1.C
DA	av1	a	a	a	av1	av1
COP	av1	a	a	a	av1	av1
ACI	av1	a	a	a	av1	av1
PVG-RT1^{a} (DA)	av1	a	a	a	av1	av1
LEW.1AV1 (DA)	av1	a	a	a	av1	av1
LEW	l	l	l	l	l	l
LEW.1N (BN)	n	n	n	n	n	n
LEW.1A (AVN)	a	a	a	a	a	a
LEW.1W (WP)	u	u	u	u	u	u
LEW.1AR1	r2	a	u	u	u	u
LEW.1AR2	r3	a	a	a	u	u
LEW.1WR1	r4	u	u	u	a	a
LEW.1WR2	r6	u	a	a	a	a
PVG.R8	r8	a	u	u	u	u
BN	n	n	n	n	n	n

Tafel 1. Ingezüchtete Rattenstämme. * = Name des Spender Stamms in Klammern

Publikation 1

Weissert, R., Svenningsson, A., Lobell, A., de Graaf, K.L., Andersson, R., and Olsson, T. (1998). Molecular and genetic requirements for preferential recruitment of TCRBV8S2^{+} T cells in Lewis rat experimental autoimmune encephalomyelits. ***J. Immunol. 160****, 681-690.*

Man hatte die Beobachtung gemacht, dass bei der mit MBP induzierten experimentellen autoimmunen Enzephalomyelitis (EAE) bei LEW (RT1^{l}) Ratten und der PL/J (H-2^{u}) Maus eine präferentielle Aktivierung und Expansion von enzephalitogenen T Zellen, welche die T Zellrezeptor variable Kette 8.2 (TZRBV8S2) tragen, auftreten (Zamvil et al., 1988; Acha-Orbea et al., 1988; Urban et al., 1988; Burns et al., 1989; Chluba et al., 1989). Außerdem fand man, dass bei einigen MS Patienten bestimmte TZRBV Ketten übermäßig häufig auf T Zellen im Blut und in Läsionen im ZNS zu finden sind (Kotzin et al., 1991; Oksenberg et al., 1993; Musette et al., 1996; Hafler et al., 1996). Man hatte aus diesen Beobachtungen geschlossen, dass mit hoher Wahrscheinlichkeit bei MS Patienten enzephalitogene T Zellen, das heißt T Zellen, welche die MS auslösen und aufrechterhalten, bestimmte TZRBV Ketten überrepräsentierten. Man hatte große therapeutische Hoffnungen gehegt, dass durch Depletion oder Modulation dieser T Zellen, Krankheit unterdrückt werden könnte, wie dieses in den EAE Modellen der Fall war (Zamvil and Steinman, 1990).

Wir hatten uns zur Aufgabe gemacht, die molekularen Mechanismen zu untersuchen, die zu einer präferentiellen Aktivierung und Expansion von TZRBV8S2 positiven Zellen führen. Dazu haben wir LEW Ratten mit dem enzephalitogenen heterologen $MBP_{Guinea\ Pig\ (GP)}$63-88 und dem autologen MBP_{RAT}63-88 immunisiert und in peripheren lymphoiden Organen und im ZNS zytofluorometrisch untersucht, wann es zu einer präferentiellen Aktivierung von TZRBV8S2 Zellen kommt (Bild 3). Die beiden Peptide MBP_{GP}63-88 und MBP_{RAT}63-88 unterscheiden sich nur in einem Aminosäureaustausch in Position 79, MBP_{GP}63-88 (S_{79}) und MBP_{RAT}63-88 (T_{79}). Beide Peptide binden gleich gut an das restringierende MHC RT1.B^{l} Allel. LEW Ratten erkrankten nach Immunisierung mit beiden Peptiden. Interessanterweise zeigten nur LEW Ratten, welche mit dem heterologen MBP_{GP}63-88 immunisiert worden waren, eine präferentielle Aktivierung und Expansion von TZRBV8S2 positiven T Zellen. Die MHC kongenen Rattenstämme LEW.1W (RT1^{u}) und LEW.1AV1 (RT1^{av1}) zeigten keine präferentielle Aktivierung und Expansion von

TZRBV8S2 positiven T Zellen. Die Depletion mit einem monoklonalen Antikörper gegen TZRBV8S2 führte nur in den MBP_{GP}63-88-immunisierten LEW Ratten, jedoch weder in mit MBP_{RAT}63-88 immunisierten LEW Ratten, noch in mit MBP_{GP}63-88 immunisierten LEW.1AV1 Ratten zu einer Reduktion der Krankheit. Diese Befunde zeigten, dass die selektive Aktivierung und Expansion von T Zellen, welche TZRBV8S2 Ketten tragen, aufgrund des LEW Ratten $RT1.B^{l}$ MHC Allels sowie dem heterologen MBP_{GP}63-88 verursacht wird.

- MBP_{GP}63-88
  AARTTHYGSLPQKSQR**S**QDENPVVHF *Fremd*

  RT1.B (DQ)
- MBP_{RAT}63-88
  HTRTTHYGSLPQKSQR**T**QDENPVVHF *Selbst*

- MBP89-101
  VHFFKNIVTPRTP **RT1.D (DR)** *Selbst*

Bild 3. Autologes (T_{79}) und heterologes (S_{79}) MBP 63-88 und die in der $RT1.B^{l}$ Bindungsgrube gebundenen Sequenzen von MBP (Reizis et al., 1996; Wauben et al., 1997).

Diese Befunde weisen darauf hin, dass die Avidität von TZRBV8S2 für die $RT1.B^{l}$/MBP_{GP}63-88-Komplexe um ein Vielfaches höher ist, als für die $RT1.B^{l}$/MBP_{RAT}63-88-Komplexe. Die Ursache dafür liegt mit hoher Wahrscheinlichkeit an zentralen Toleranzmechanismen im Thymus (Sprent and Kishimoto, 2001). Andere Arbeitsgruppen haben mittels Langzeit T Zelllinien diese Befunde bestätigt und die zentrale Hypothese der aviditätsbedingten Expansion bestimmter TCRBV Ketten gestützt (Mor et al., 2000).

Kürzlich wurden ähnliche Befunde in einem Mausmodell für $CD8^{+}$ T Zellen etabliert. Auch diese Daten besagen, dass die Regulation von Immunantworten gegenüber Fremdantigenen im Vergleich zu Selbstantigenen durch eine erhöhte Avidität des TZR gegenüber Fremdantigen/MHC Komplexen reguliert wird (de Visser et al., 2001).

Unsere Daten führten zu der Hypothese, dass ein restringiertes TZRBV T Zellrepertoire bei einigen MS Patienten auf Grund von einem molekularen Mimikry auftreten kann (Wucherpfennig and Strominger, 1995). Dies bedeutet, dass die MS durch exogene Faktoren ausgelöst wird, welche auf dem Boden einer genetischen Prädisposition ihren Einfluss ausüben. Des weiteren zeigen die Daten jedoch auch die Schwierigkeiten auf, T Zelldepletionen von T Zellen, die bestimmte TZRBV Ketten tragen, bei MS Patienten mit therapeutischem Erfolg durchzuführen, da eine Variabilität der präsentierenden MHC Moleküle und der die Krankheit induzierenden Umweltfaktoren besteht.

Kürzlich wurde berichtet, dass bei MS Patienten innerhalb von ZNS Läsionen Expansionen von CD8 positiven T Zellen vorhanden sind, welche die gleichen TZR Ketten und CDR3 Sequenzen bei unterschiedlichen Patienten exprimieren (Babbe et al., 2000). Auch diese Expansionen könnten durch den Kontakt mit einem Fremdantigen wie einem viralen Antigen bedingt sein. Diesen Befunden wird weiter nachgegangen und die funktionelle Charakterisierung dieser T Zellen vorgenommen. So könnte es sich sowohl um pathogene und zytotoxische T Zellen als auch um regulatorische krankheitssupprimierende T Zellen handeln (Mustafa et al., 1994).

Publikation 2

Weissert, R., Lobell, A., de Graaf, K.L., Eltayeb, S.A., Andersson, R., Olsson, T., and Wigzell, H. (2000). Protective DNA vaccination against organ-specific autoimmunity is highly specific and discriminates between single amino acid substitutions in the peptide autoantigen. ***Proc. Natl. Acad. Sci. USA 97****, 1689-1694.*

Wir hatten gezeigt, dass mittels intramuskulär injizierter nackter DNS, welche für autoantigene MBP Sequenzen kodiert, die MBP-induzierte EAE unterdrückt werden kann (Lobell et al., 1998). In Arbeiten war gezeigt worden, dass TZR in einem hohen Maße in ihrer Fähigkeit zur spezifischen Antigenerkennung degeneriert sein können (Hemmer et al., 1997; Mason 1998). Wir waren daran interessiert zu untersuchen, ob der Effekt der Vakzinierung mit nackter DNS spezifisch ist, oder ob ein therapeutischer Effekt auch gegen enzephalitogene T Zellen anderer Spezifität auftritt.

Wir haben DNS Vakzine konstruiert, welche entweder die Sequenzen MBP_{GP}68-85 (S_{79}) oder MBP_{RAT}68-85 (T_{79}) kodierten. Nach der intramuskulären Vakzinierung, LEW Ratten haben wir entweder mit dem Peptid MBP_{GP}68-85 (S_{79}) oder MBP_{RAT}68-85 (T_{79}) immunisiert. Dabei zeigte sich eine hohe Spezifität der Protektion gegenüber EAE, welche mit dem Austausch von Serin nach Threonin in der DNS Vakzine assoziiert war. Mittels einer DNS Vakzine, welche die enzephalitogene Sequenz MBP89-101 kodierte, konnte dargestellt werden, dass die Protektion auch bei dieser Sequenz spezifisch war und kein Schutz gegen die mit MBP_{GP}68-86 (S_{79}) oder MBP_{RAT}68-86 (T_{79}) induzierte EAE auftrat.

Diese Befunde weisen darauf hin, dass das T Zellrepertoire in Bezug auf die Erkennung enzephalitogener Sequenzen eine hohe Spezifität aufweist. Außerdem zeigen diese Befunde, dass die Anwendung der nackten DNS Vakzine einen spezifischen Effekt auf das enzephalitogene Autoantigen-restringierte T Zellrepertoire hat. Die Protektion ist dabei nicht, wie dieses bei der oralen und nasalen Toleranz gezeigt wurde, durch eine *bystander* Supprimierung bedingt (Weiner, 1997).

Kürzlich wurde in Mäusen gezeigt, dass die Ko-Immunisierung mit Interleukin 4 (IL-4) zu einer verbesserten Protektion der DNS Vakzine führen kann (Garren et al., 2001). Wir hatten in Ratten gezeigt, dass die Ko-Immunisierung mit IL-4 keinen Einfluss auf den Erfolg der DNS Vakzinierung hat (Lobell et al., 1999). In unseren Händen wirkte

dagegen ein zusätzlich verabreichtes GM-CSF Konstrukt protektiv (Lobell et al., 1999). Ein möglicher Effekt der DNS Vakzinierung wird durch Einfluss auf die Antigenpräsentation von dendritischen Zellen vermittelt, die in den verschiedenen Spezies unterschiedlich auf die mit dem Autoantigen verabreichten Wachstumsfaktoren reagieren.

Des weiteren wurde gezeigt, dass verschiedene Mausstämme in unterschiedlicher Weise auf die DNS Vakzinierung mit autoantigenen Sequenzen reagieren. So entwickelten SJL/J Mäuse eine stärkere Erkrankung nach DNS Vakzinierung mit MOG 1-218 (Bourquin et al., 2000) (Tafel 2). Unter Zusammenschau aller im EAE Modell durchgeführten Studien mit DNS Vakzinen wurde deutlich, dass der Erfolg einer Vakzinierung bei Autoimmunerkrankungen von dem genetischen Hintergrund, dem Konstrukt und der Wahl des Autoantigens bzw. der enzephalitogenen Determinante und dem Zeitpunkt der Applikation der nackten DNS abhängig ist (Tafel 2).

DNS Konstrukt	**Stamm**	**Therapieeffekt**	**Referenz**
TCRBV8S2	PL/J (H-2^u)	Suppression	(Waisman et al., 1996)
MBP_{gp}68-85 verbunden mit Fc von IgG	LEW (RT1^l)	Suppression	(Lobell et al., 1998; 1999; Weissert et al., 2000)
PLP, PLP 139-151, PLP 178-191	SJL (H-2^s)	Exazerbation	(Tsunoda et al., 1998)
PLP 139-151	SJL (H-2^s)	Suppression	(Ruiz et al., 1999)
PLP	SJL (H-2^s)	4 Wochen nach Vakzinierung Exazerbation	(Selmaj et al., 2000)
		10 Wochen nach Vakzinierung Suppression	
MBP_{rat}68-85 und MBP 89-101 verbunden mit Fc von IgG	LEW (RT1^l)	Suppression	(Weissert et al., 2000)
MOG 1-218	SJL (H-2^s)	Exazerbation	(Bourquin et al., 2000)

Tafel 2. Aufstellung der therapeutischen Effizienz bei nackter DNS Vakzinierung.

Ein Vorteil der DNS Vakzinierung liegt darin, dass längerfristige immunologische Veränderungen durch Persistenz der als DNS verabreichten Sequenzen erreicht werden. Aufgrund der hohen Spezifität des therapeutischen Effektes der durch die DNS Vakzinierung verabreichten Sequenzen, ist eine genaue Kenntnis der enzephalitogenen Determinanten notwendig. Daher müssen sowohl im Tiermodell als auch beim Menschen weitere intensive Anstrengungen unternommen werden, enzephalitogene Sequenzen von Myelinbestandteilen zu bestimmen.

Die Vakzinierung mit nackter DNS stellt ein neues therapeutisches Prinzip dar, welches auch zur Behandlung von Autoimmunerkrankungen beim Menschen weiter exploriert werden sollte (Steinman, 2001). Bei einer Vielzahl von Vakzinierungsstrategien gegen infektiöse Erkrankungen ist dieses Prinzip bereits in der klinischen Prüfung (Lai and Bennett, 1998; Ada, 2001). Die Herstellungskosten für nackte DNS Vakzine sind gering und die DNS ist sehr gut haltbar.

Publikation 3

Weissert, R., Wallström, E., Storch, M.K., Stefferl, A., Lorentzen, J., Lassmann, H., Linington, C., and Olsson, T. (1998). MHC haplotype-dependent regulation of MOG-induced EAE in rats. ***J. Clin. Invest. 102****, 1265-1273.*

In der EAE wurden eine Vielzahl von Untersuchungen zur Pathogenese und Therapie der MS durchgeführt. Die meisten der bisher verwendeten MS Modelle zeichneten sich jedoch dadurch aus, dass das typische klinische Spektrum der MS mit unterschiedlichen klinischen Verläufen, sowie die Läsionspathologie mit Entmarkung und axonalem Verlust, nicht reproduziert wurde (Wekerle et al., 1994). In der vorliegenden Arbeit haben wir ein neues Modell der MS etabliert, welches sowohl eine Vielzahl von klinischen Verläufen der MS, als auch ZNS-Läsionen mit Entzündung, Entmarkung und axonalem und neuronalem Verlust reproduziert (Storch et al., 1998; Kornek et al., 2000; 2001; Meyer et al., 2001). Dabei haben wir Myelin-Oligodendrozyten-Glykoprotein (MOG), welches auf der Myelinscheide im ZNS in geringer Menge exprimiert wird, als Enzephalitogen verwendet (Bernard et al., 1997).

Die einzig sicheren in großen Assoziationsstudien bei der MS definierten Risikofaktoren stellen MHC Klasse II Gene dar (Oksenberg et al., 2001). Wir waren daran interessiert den MHC Einfluss in diesem neuen, verbesserten Modell der MS zu untersuchen. Wir haben deshalb eine große Anzahl von ingezüchteten Rattenstämmen mit der extrazellulären MOG Domäne der Ratte immunisiert und sowohl den klinischen und histopathologischen Phänotyp, als auch die Immunantworten untersucht.

Dabei zeigte sich, dass der MHC Haplotyp keinen Alles-oder-Nichts Effekt auf die Krankheit ausübt, sondern eine Haplotypen-abhängige Graduierung der Krankheitsausprägung bedingte (Tafel 1 und 3). Zudem fanden sich abhängig vom MHC Haplotypen unterschiedliche klinische Verläufe und unterschiedliche Läsionstypen (Tafel 3). Diese Ergebnisse waren unerwartet und neu. Immunantworten gegen die extrazelluläre MOG Domäne früh im Krankheitsverlauf waren in den hoch-suszeptiblen Rattenstämmen stärker ausgeprägt als in den niedrigsuszeptiblen oder resistenten Rattenstämmen.

Stamm	Verlauf
LEW	Keine Erkrankung
LEW.1W	Primär progredient
LEW.1A	Chronisch
LEW.1AV1, DA	Schubförmig
LEW.1N	Hyperakut, chronisch

Tafel 3. Krankheitsverläufe in der MOG-EAE nach Immunisierung mit unlöslichem MOG.

Wir untersuchten mittels intra-MHC kongener Rattenstämme, welcher Teil des MHC vornehmlich die Erkrankung bedingt (Tafel 1). Dabei zeigte sich, dass der Hauptsuszeptibilitätslokus innerhalb der MHC Klasse II Region liegt. Zudem nehmen auch MHC Klasse I Gene und Gene innerhalb des MHC, die telomer zur Klasse II Region gelegen sind, Einfluss auf die Erkrankung und die Läsionsgenese (Bild 1). Wir haben den Einfluss von nicht-MHC Genen auf einen suszeptiblen MHC Haplotypen in verschiedenen ingezüchteten Rattenstämmen untersucht und konnten darstellen, dass eine übergeordnete genetische Regulation durch nicht-MHC Gene ausgeübt wird. In weiterführenden Untersuchungen haben wir begonnen, diese zu analysieren (Dahlman et al., 1999; Jagodic, 2001).

Wir konnten mittels Titrationsstudien in suszeptiblen Rattenstämmen zeigen, dass die für die Auslösung der Erkrankung notwendige Antigenmenge sich stark unterscheidet. So konnten in den hoch-suszeptiblen LEW.1N Ratten mit 1 μg MOG ein chronischer Krankheitsverlauf und ZNS Läsionen ausgelöst werden, während dieses bei der LEW.1AV1 Ratte nur ab einer MOG Dosis von 20 μg möglich war. Diese Daten unterstreichen, dass bei Individuen, welche bestimmte MHC Haplotypen tragen, kleinste Antigenmengen hypothetisch in der Lage sind, Krankheit auszulösen.

Außerdem konnte in der Arbeit dargestellt werden, dass kein MHC Haplotyp eine absolute Protektion vor Erkrankung gewährt, sondern dass abhängig vom Autoantigen bestimmte MHC Haplotypen Krankheit auslösen können. So sind LEW Ratten nach Immunisierung mit MOG vor Erkrankung geschützt, sie werden jedoch sehr krank nach Immunisierung mit MBP (Tafel 4).

Haplotypus	MBP	PLP	MOG_{1-125}
$RT1^{l}$	+++ (Happ et al., 1988)	+ (Yamamura et al., 1986)	- (Weissert et al., 1998)
$RT1^{av1}$	+++ (Lorentzen et al., 1995; Stepaniak et al., 1995)	+++ (Stepaniak et al., 1995)	+++ (Weissert et al., 1998)
$RT1^{u}$	n.b.	n.b.	+ (Weissert et al., 1998)
$RT1^{n}$	- (Happ et al., 1988)	n.b.	++++ (Weissert et al., 1998)

Tafel 4. MHC Haplotypen-vermittelte Suszeptibilität in Kontext mit Autoantigen. n.b. = nicht bestimmt.

Die Arbeit liefert starke Argumente für die primäre Immunpathogenese der MS auf dem Boden einer genetischen Prädisposition. Des weiteren vermittelt sie ein grundlegendes Verständnis zu genetischen Suszeptibilitätsfaktoren im Zusammenspiel mit Umweltfaktoren und liefert ein neues und verbessertes Verständnis zu MHC Einflüssen bei der MS. Besonders hervorzuheben ist der Befund, dass der MHC Haplotypus den Krankheitsverlauf bestimmt. Ob der MHC Haplotypus auch bei MS Kranken einen Einfluss auf den klinischen Verlauf hat, muss in großen MS Kollektiven untersucht werden.

Publikation 4

Weissert, R., de Graaf, K.L., Storch, M.K., Barth, C., Linington, C., Lassmann, H., and Olsson, T. (2001). MHC class II-regulated central nervous system autoaggression and T cell responses in peripheral lymphoid tissue are dissociated in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis. ***J. Immunol. 166****, 7588-7599.*

Wir haben im MOG Modell mit 18 Aminosäuren langen Peptiden die Notwendigkeiten für die Auslösung der Erkrankung auf der T- und B Zellebenen untersucht. Dabei konnten wir darstellen, dass die Sequenz MOG 91-108 in suszeptiblen Rattenstämmen enzephalitogen ist. Interessanterweise konnten in den lymphoiden Organen nur bei LEW.1AV1 Ratten die autoreaktiven MOG 91-108-spezifischen T Zellen detektiert werden. In der LEW.1N Ratte konnte ex-vivo keine T Zellantwort gegen MOG 91-108 in den lymphoiden Organen gemessen werden, jedoch wie auch bei der LEW.1AV1 Ratte, innerhalb des ZNS (Tafel 5). Dieses deutet darauf hin, dass das Zielorganmilieu bzw. die Antigenpräsentation innerhalb des Zielorgans die Suszeptibilität gegenüber der Erkrankung bestimmen. Ähnliche Befunde wurden in der Zöliakie erhoben, die mit den MHC Klasse II Allelen HLA-DQ2 und DQ8 assoziert ist. Dort wurde gezeigt, dass die Immunantwort im Darm sich aufgrund der Expression von Transglutaminase fundamental von der im Blut unterscheidet (Molberg et al., 1998).

Stamm	Dominante T Zell-Determinante	Enzephalitogene T Zell-Determinante
LEW	MOG 37-54	Keine
LEW.1W	Keine	Keine
LEW.1AV1	MOG 73-90	
	MOG 91-108	MOG 91-108
	MOG 97-114	MOG 97-114
LEW.1N	MOG 19-36	
		MOG 91-108
		MOG 97-114

Tafel 5. Dominante und enzephalitogene Determinanten in der MOG-EAE (Weissert et al., 2001).

Es war für uns von großem Interesse, das Bindungsverhalten von immunogenen und enzephalitogenen Peptiden an aufgereinigte MHC Moleküle in den Rattenmodellen zu bestimmen. So gibt es Hinweise darauf, dass neben gut bindenden Peptiden auch sehr schlecht bindende Peptide Krankheit auslösen können (Wall et al., 1992; Fairchild et al., 1993; Wucherpfennig et al., 1994; Liu et al., 1995; Greer et al., 1996; Weissert et al., 1998; de Graaf et al., 1999). Wir hatten die Hypothese, dass im Allgemeinen, aufgrund der Notwendigkeit der seriellen Triggerung von TZR zur Aktivierung von T Zellen durch wenige Peptid-MHC Komplexe, enzephalitogene Determinanten zu den mittelgut-bis gut bindenden Peptiden gehören sollten (Valitutti et al., 1995; Valitutti and Lanzavecchia, 1997). Ein hoch-affin bindendes Peptid hat im Vergleich zu einem niedrig-affin bindenden Peptid eine höhere Wahrscheinlichkeit auf einem MHC Molekül präsentiert zu werden und kann daher eher zur Aktivierung von autoreaktiven T Zellen führen.

Wir haben zwölf Aminosäuren überlappende 18 Aminosäuren lange Peptide der extrazellulären MOG-Sequenz 1-125 in ihrem Bindungsverhalten an affinitätschromatographisch aufgereinigte RT1.B^{l}, RT1.B^{u}, RT1.B^{a}, RT1.B^{n}, RT1.D^{l}, RT1.D^{u}, RT1.D^{a} und RT1.D^{n} Moleküle untersucht (das RT1.B Molekül der Ratte ist dem DQ Molekül des Menschen ähnlich und das RT1.D Molekül dem DR Molekül). Die immunogenen und enzephalitogenen Sequenzen gehörten zu den mittelguten bis guten Bindern, wobei mehr Peptide in der Lage waren an MHC Moleküle zu binden als eine Immunantwort auszulösen. Unterschiede in den Bindungseigenschaften zwischen immunogenen und/oder enzephalitogenen Determinanten wurden nicht gefunden. Die unterschiedlichen MHC Allele unterschieden sich deutlich in ihren Bindungseigenschaften gegenüber den MOG Peptiden. Interessanterweise korrelierte die Anzahl der bindenden Peptide mit der Heftigkeit und Chronizität der Erkrankung. Dieses könnte durch die Präsentation einer höheren Anzahl von MOG Determinanten bedingt sein. Die enzephalitogenen Sequenzen gehörten zu den mittelguten bis guten Bindern (Tafel 6).

Haplotyp (Stamm)	Enzephalitogen	Restriktion	IC_{50} (µM)	Referenzen
$RT1^{l}$ (LEW)	MBP_{gp}72-85	B^{l}	2,5	(Weissert et al., 1998)
	MBP 87-99	D^{l}	0,02	(de Graaf et al., 1999)
$RT1^{av1}$ (DA, LEW.1AV1)	MBP 87-99	B^{a}	0,06	(de Graaf et al., 1999)
	MOG 91-108	B^{a}	9,5	(Weissert et al., 2001)
$RT1^{n}$ (BN, LEW.1N)	MOG 91-108	D^{n}	0,03	(Weissert et al., 2001)
$H\text{-}2^{s}$ (SJL/J)	MBP 81-100	$I\text{-}A^{s}$	0,36	(Wall et al., 1992)
	PLP 100-119	$I\text{-}A^{s}$	1,24	(Greer et al., 1996)
	PLP 139-151	$I\text{-}A^{s}$	0,04	(Greer et al., 1996)
	PLP 178-191	$I\text{-}A^{s}$	0,74	(Greer et al., 1996)
$H\text{-}2^{u}$ (PL/J)	MBP Ac1-9	$I\text{-}A^{u}$	> 100	(Fairchild et al., 1993; Liu et al., 1995)
HLA-DR2 transgene Mäuse	MBP 84-102	DRB1*1501	0,004	(Wucherpfennig et al., 1994; Madsen et al., 1999)
HLA-DR4 transgene Mäuse	MOG 91-108	DRB1*0401	0,3	(Forsthuber et al., 2001)

Tafel 6. Peptide/MHC Klasse II Bindungseigenschaften und Enzephalitogenität. Die IC_{50} gibt die Stärke der MHC-Peptidinteraktion an. Je niedriger die IC_{50}, desto besser bindet das Peptid an das MHC Klasse II Molekül.

Unter Zusammenschau weiterer von uns in der MBP-EAE der Ratte erhobener Daten (de Graaf et al., 1999) zeigen diese Daten, dass im Regelfall enzephalitogene Determinanten auch beim Menschen zu den gut bindenden Determinanten zählen werden (Tafel 6). Die MHC Haplotypen/Autoantigen-assoziierten Peptidbindungsdaten legen nahe, dass möglicherweise MHC Klasse II Haplotypen-Assoziation in Zusammenhang mit der Promiskuität des Bindungsverhaltens autoantigener Sequenzen an MHC Moleküle korreliert ist. Des weiteren unterstreichen die Ergebnisse die Komplexität der Autoimmungenese in Bezug auf die Immunantwort im Zielorgan. Die Untersuchungen legen nahe, dass bei MS Patienten die Immunantwort im Zielorgan charakterisiert werden muss, da diese viel aussagekräftiger ist als die Immunantwort in peripheren lymphoiden Organen oder im Blut.

Verschiedene Autoren haben Hinweise darauf, dass Antikörper gegen MOG pathogen sein könnten, indem sie die Demyelinisierung begünstigen (Lassmann et al., 1988; Genain 1995, 1996; Reindl et al., 1999; Brehm et al., 1999; Genain et al., 1999). So konnte durch die Injektion von MOG spezifischen Antikörpern in das ZNS eine Entmarkungskrankheit ausgelöst werden (Lassmann et al., 1988). In ‚knock-out' Modellen in der Maus, in welchen keine B Zellen zu finden sind, wurde die Enzephalitogenität von MOG untersucht, wobei es Hinweise darauf gab, dass B Zellen für die Enzephalitogenität von kurzen Peptiden nicht notwendig sind, jedoch bei der Immunisierung mit MOG 1-125 (Hjelmstrom et al., 1998; Lyons et al., 1999). Zudem wurde MOG spezifisches Immunglobulin transgen in Mäusen überexprimiert und die Erkankungsschwere der Mäuse untersucht (Litzenburger et al., 1998). Unter Zusammenschau aller dieser Untersuchungen ist derzeit davon auszugehen, dass MOG spezifische Antikörper für die Entwicklung entmarkender Läsionen nicht notwendig sind, dass diese allerdings die Entmarkung als ‚Ko-Faktor' begünstigen können.

In unseren Untersuchungen fanden wir nach Immunisierung mit MOG 91-108 in LEW.1AV1 und LEW.1N Ratten kreuzreaktive Antikörper gegen MOG 1-125. Es fanden sich auch Antikörperantworten gegen MOG 91-108 nach Immunisierung mit MOG 1-125. Dieses deutet darauf hin, dass möglicherweise B Zellen eine Rolle bei der Antigenpräsentation zukommt und MOG 91-108 eine lineare B Zelldeterminante darstellt, die von B Zellen T Zellen präsentiert wird. Diese sogenannte ‚cognate' Erkennung von MOG 91-108 von T- und B Zellen könnte eine der Gründe für die hohe Enzephalitogenität von MOG 91-108 sein, da B Zellen aufgrund ihrer Spezifität kleinste Antigenmengen erkennen können und T Zellen präsentieren können und damit die Präsentation der Determinante MOG 91-108 erhöhen könnten (Jenkins et al., 2001). Auf der anderen Seite haben B Zellen ein hohes Potential zur Toleranzinduktion von $CD4^+$ T Zellen (Glynne et al., 2000). Dieses könnte bedingen, dass in LEW.1N Ratten in peripheren lymphoiden Organen keine T Zellantwort gegen MOG 91-108 gemessen werden konnte. Derzeit führen wir weiterführende Untersuchungen zur Entschlüsselung dieser Befunde durch.

Bei der Analyse der ZNS-spezifischen Immunantwort in LEW.1AV1 und LEW.1N Ratten fanden wir mittels der quantitativen ‚real time' PCR hohe mRNA Expressionen von TNF-α, IL-4 und IFN-γ. Diese Zytokine sind in der Lage, die intrazerebrale

Immunantwort durch Aktivierung antigenpräsentierender Zellen, Diversifizierung der Immunantwort und direkter Schädigung des ZNS zu amplifizieren. So konnte mehrfach gezeigt werden, dass TNF-α in der Lage ist, Entmarkung im ZNS auszulösen (Probert et al., 2000). Unsere Befunde bezüglich der intrazerebralen Immunantwort würden in diese Richtung weisen und TNF-α einen maßgeblichen Einfluss bei der Entmarkung zuweisen.

Publikation 5

Forsthuber, T.G., Shieve, C.L., Wienhold, W., de Graaf, K.L., Spack, E.G., Sublett, R., Melms, A., Kort, J., Racke, M.K., and **Weissert, R.** (2001). T cell epitopes of human myelin oligodendrocyte glycoprotein indentified in HLA-DR4 (DRB1*0401) transgeneic mice are encephalitogenic and are presented by human B cells. ***J. Immunol. 167****, 7119-7125.*

Wir haben in einem Mausstamm, welcher HLA-DR4 (DRB1*0401) exprimiert die Immunogenität und Enzephalitogenität gegenüber MOG bestimmt. Die Spezifität DRB1*0401 ist mit der MS in einigen Populationen, wie z.B. in Sardinien assoziiert (Marrosu et al., 1988). In HLA-DR4 (DRB1*0401) transgenen Mäusen konnten wir zeigen, dass die Sequenzen MOG 91-108 und MOG 97-108 enzephalitogen sind. Humane DRB1*0401 exprimierende B Zellen konnten die extrazelluläre Domäne von MOG, MOG 1-125, als Antigen-präsentierende Zellen (APZ) T Zellen von MOG 97-108 immunisierten HLA-DR4 transgenen Mäusen präsentieren. MOG 91-108 band gut an aufgereinigte HLA-DRB1*0401 Moleküle. Dieses stützt die von uns in der Ratte erhobenen Daten bezüglich der Bindungseigenschaften von enzephalitogenen Sequenzen (Tafel 6).

Diese Daten zeigen eindrucksvoll, dass die MOG Determinante MOG 91-108 auch in einem humanisierten Mausmodell enzephalitogen ist. In LEW.1AV1 ($RT1^{av1}$), DA ($RT1^{av1}$), COP ($RT1^{av1}$), PVG-$RT1^{a}$ ($RT1^{av1}$) und LEW.1N ($RT1^{n}$) Ratten ist MOG 91-108 die primäre enzephalitogene Sequenz (Tafel 5). Auch in SJL ($H\text{-}2^{s}$) Mäusen ist diese Sequenz enzephalitogen (Amor et al., 1994). Befunde bei Marmosetten und Makaken deuten ebenso darauf hin, dass MOG 91-108 enzephalitogen ist (Kerlero de Rosbo et al., 2000; Villoslada et al., 2001). Diese Befunde zeigen an, dass MOG 91-108 möglicherweise auch beim Menschen eine enzephalitogene Determinante darstellt. Aus verständlichen Gründen konnte beim Menschen bisher experimentell nicht geklärt werden, welche MOG Sequenzen enzephalitogen sind. Derzeit untersuchen wir die Gründe für die Enzephalitogenität von MOG 91-108.

Wir zeigen, dass die MOG Sequenz 91-108 in verschiedenen Spezies und im Kontext mit verschiedenen MHC Klasse II Haplotypen enzephalitogen ist. Diese Daten unterstreichen, dass dieser Sequenz auch im Kontext mit der MS Beachtung geschenkt werden sollte. Besonders hervorzuheben ist jedoch, dass die in der

Peripherie gemessene T Zellantwort möglicherweise nicht zur Bestimmung von enzephalitogenen Determinanten beim Menschen ausreicht, da in der LEW.1N Ratte zwar MOG 91-108 sehr enzephalitogen ist, jedoch keine Immunantwort in peripheren lymphoiden Organen gegen dieses Peptid gemessen werden kann. In Publikation 7 haben wir bei MS Patienten und Kontrollpersonen das T Zellrepertoire gegenüber MOG 1-218 untersucht. Wir konnten keine signifikanten Reaktivitäten gegenüber MOG 91-108 finden. Diese Untersuchungen zeigen, dass humanisierte Tiermodelle herangezogen werden sollten, um enzephalitogene MHC Klasse II restringierte Determinanten beim Menschen zu bestimmen.

Publikation 6

de Graaf, K.L., Barth, S., Herrmann, M.M., Storch, M.K., Otto, C., Olsson, T., Melms, A., Jung, G., Wiesmuller, K.H., and **Weissert, R.** (2004) MHC class II isotype- and allele-specific attenuation of experimental autoimmune encephalomyelitis. ***J. Immunol. 173***, 2792-2802

Wir haben den fundamentalen Einfluss der MHC Klasse II Region auf die Regulation der MOG-EAE gezeigt (Weissert et al., 1998; Weissert et al., 2001). Als therapeutisches Prinzip wollten wir die Blockade von krankheitsassoziierten MHC Klasse II Molekülen untersuchen (Bolin et al., 2000). Wir haben deshalb mittels randomisierter, kombinatorischer Peptidbibliotheken das Peptidbindungsmuster des RT1.D^n Moleküls bestimmt (Tafel 7) (Fleckenstein et al., 1999). Bisher waren bis auf das RT1.B^l Molekül der LEW Ratte keine Ligandenmotive oder Peptidbindungsmuster der Ratte bekannt (Reizis et al., 1996; Wauben et al., 1997).

Ac-AX$_n$-NH$_2$	**Ac-DX$_n$-NH$_2$**	**Ac-OX-NH$_2$**	**Ac-YX$_n$-NH$_2$**
Ac-AXXXXXXXX-NH$_2$	Ac-DXXXXXXXX-NH$_2$	…	Ac-YXXXXXXXX-NH$_2$
Ac-XAXXXXXXX-NH$_2$	Ac-XDXXXXXXX-NH$_2$	…	Ac-XYXXXXXXX-NH$_2$
Ac-XXAXXXXXX-NH$_2$	Ac-XXDXXXXXX-NH$_2$	…	Ac-XXYXXXXXX-NH$_2$
…	…	…	…
Ac-XXXXXXXAX-NH$_2$	Ac-XXXXXXXDX-NH$_2$	…	Ac-XXXXXXXYX-NH$_2$
Ac-XXXXXXXXA-NH$_2$	Ac-XXXXXXXXD-NH$_2$	…	Ac-XXXXXXXXY-NH$_2$

Tafel 7. Struktur der aminoterminal azetylierten und carboxyterminal amidierten nonameren Peptidbibliotheken.

Mittels der gewonnenen Daten haben wir an die RT1.D^n Moleküle mit hoher Affinität bindende Peptide vorhergesagt und diese experimentell in Bindungsassays überprüft. Mit vier der am besten bindenden Peptide haben wir LEW.1N (RT1^n) Ratten aktiv immunisiert. Dabei zeigte sich, dass alle vier getesteten Peptide starke T Zellantworten induzierten. Mit dem Peptid p17 mit der höchsten Affinität und der niedrigsten IC$_{50}$ von 2 nM konnten wir in Koimmunisierungsexperimenten die MOG-EAE in LEW.1N Ratten sowohl in der MOG 91-108 und der MOG 1-125 induzierten EAE verhindern. Als Kontrollpeptid setzten wir ein Peptid mit einer hohen IC$_{50}$ von über 100 µM ein. Dieses Peptid war nicht dazu in der Lage die MOG-EAE zu

unterdrücken. Zu Vergleichsstudien haben wir auch mit Copaxone (COP-1) in der mit MOG 1-125 immunisierten EAE koimmunisiert. Diese Koimmunisierung führte nicht zur Krankheitssuppression. Es fanden sich hohe Zahlen von T Zellen, welche p17 reaktiv waren, was darauf hinweist, dass p17 starke immunmodulatorische Eigenschaften haben könnte.

Unter Zusammenschau der Experimente waren wir in der Lage mit einer hohen Affinität an MHC Klasse II Moleküle bindende Peptide zu bestimmen (Tafel 8). Bisher waren solche, sogenannten MHC blockierenden Peptide, durch die Variation von bekannten Peptiden von Autoantigenen oder Fremdantigenen bestimmt worden. Mittels der randomisierten, kombinatorischen Peptidbibliotheken haben wir Peptide bestimmt, die in höchstem Maße darauf optimiert sind, an MHC Klasse II Moleküle zu binden. Als therapeutisches Prinzip ist dieser Ansatz sehr sinnvoll, da selektiv Autoimmunitäts-assoziierte MHC Moleküle blockiert werden können (Bild 4).

Zum einen wird in der Arbeit ein Weg zur Bestimmung von hoch-affin an MHC Moleküle bindende Peptide aufgezeigt. Zum anderen konnte mittels des an RT1.D^n-hoch-affin bindenden Peptides p17, die MOG-EAE unterdrückt werden. Bisher ist noch nicht geklärt, ob der therapeutische Effekt alleine durch die Blockade der MHC Klasse II Bindungsstelle (Kompetition bei der Antigenpräsentation) oder auch durch die Immunogenität von p17 bedingt ist. So könnte einer der therapeutischen Effekte von p17 darauf beruhen, dass ein Einfluss auf das T Zellrepertoire ausgeübt wird, welcher durch eine höhere Avidität der p17 reaktiven T Zellen, das Autoantigen-restringierte MOG spezifische T Zellrepertoire unterdrückt (McHeyzer-Williams and Davis, 1995). Die Daten sind sehr erfolgsversprechend und sind die Grundlage für die weitere Exploration diesen Prinzips.

Neu an der Arbeit ist die Verhinderung der EAE mit einem Peptid, welches darauf optimiert wurde, sehr gut an die Autoimmunitäts-assoziierten MHC Moleküle zu binden und welches keine Sequenzhomologien zu dem autoantigenen Peptid hat. Bei der mit MBP induzierten EAE in der LEW Ratte wurden kürzlich ähnliche Ansätze beschrieben. Dabei wurden Peptide auf Basis des enzephalitogenen MBP Peptides und der Kenntnis der Kristallstruktur von HLA-DR2 hergestellt, die auf Bindung an die MHC Klasse II Bindungstaschen optimiert sind. Ein solches Peptid war in der Lage, die EAE zu unterdrücken (Ruiz et al., 2001).

Peptiddesign

- Enzephalitogen (Gautam, 1995)
- Modifiziertes Enzephalitogen (TZR Antagonist, altered peptide ligand [APL]) (Wraith et al., 1989; Karin et al., 1994; Nicholson et al., 1995; Samson and Smilek, 1995; Brocke et al., 1996; Nicholson et al., 1997) (Bild 4)
  - TZR Kontaktstelle
  - MHC Ankerstelle zur Verbesserung der MHC Klasse II Bindung
  - TZR Kontaktstelle und MHC Klasse II Ankerstelle
- Nicht-selbst Peptid, welches am gleichen Restriktionsmolekül wie das Enzephalitogen bindet (Wauben et al., 1994)
- Peptide, die auf Bindungsdaten basieren (Ruiz et al., 2001)

Verabreichung des modulierenden Peptides

- Durch Koimmunisation mit dem Enzephalitogen
- Bevor oder/und nach Immunisierung
- Subcutan, intraperitoneal vor oder/und nach der Immunisierung
- Nasal/oral vor oder/und nach der Immunisierung

Mechanismen (Kamradt and Mitchison, 2001)

- Anergie/Deletion
- Beeinflussung des Zytokinmusters
  - Th1/Th2 Verschiebung
  - TGF-β vermittelte *bystander* Unterdrückung
  - Reduktion der Produktion pathogener Zytokine wie TNF-α und IFN-γ
- TZR Antagonismus
- Kompetition für die MHC Klasse II Bindung
- Klonale Dominanz des Kompetitorpeptides, wenn dieses immunogen ist

Tafel 8. Modulation der EAE durch Peptide

Im Vergleich zu ‚Altered Peptide Ligands (APL) (Evavold and Allen, 1991; Sloan-Lancaster et al., 1993), die selektiv nur bestimmte T Zellpopulationen beeinflussen und modulieren, bieten die hoch-affin an MHC Klasse II Moleküle bindenden Peptide den Vorteil, das gesamte durch das ‚blockierte' MHC Klasse II Molekül restringierte T Zellrepertoire und dessen Aktivierung zu beeinflussen. Dadurch wird die Gefahr unerwünschter APL Effekte, die statt zur Suppression von autoimmunen T Zellpopulationen zur Aktivierung führen, unterdrückt (Bielekova et al., 2000).

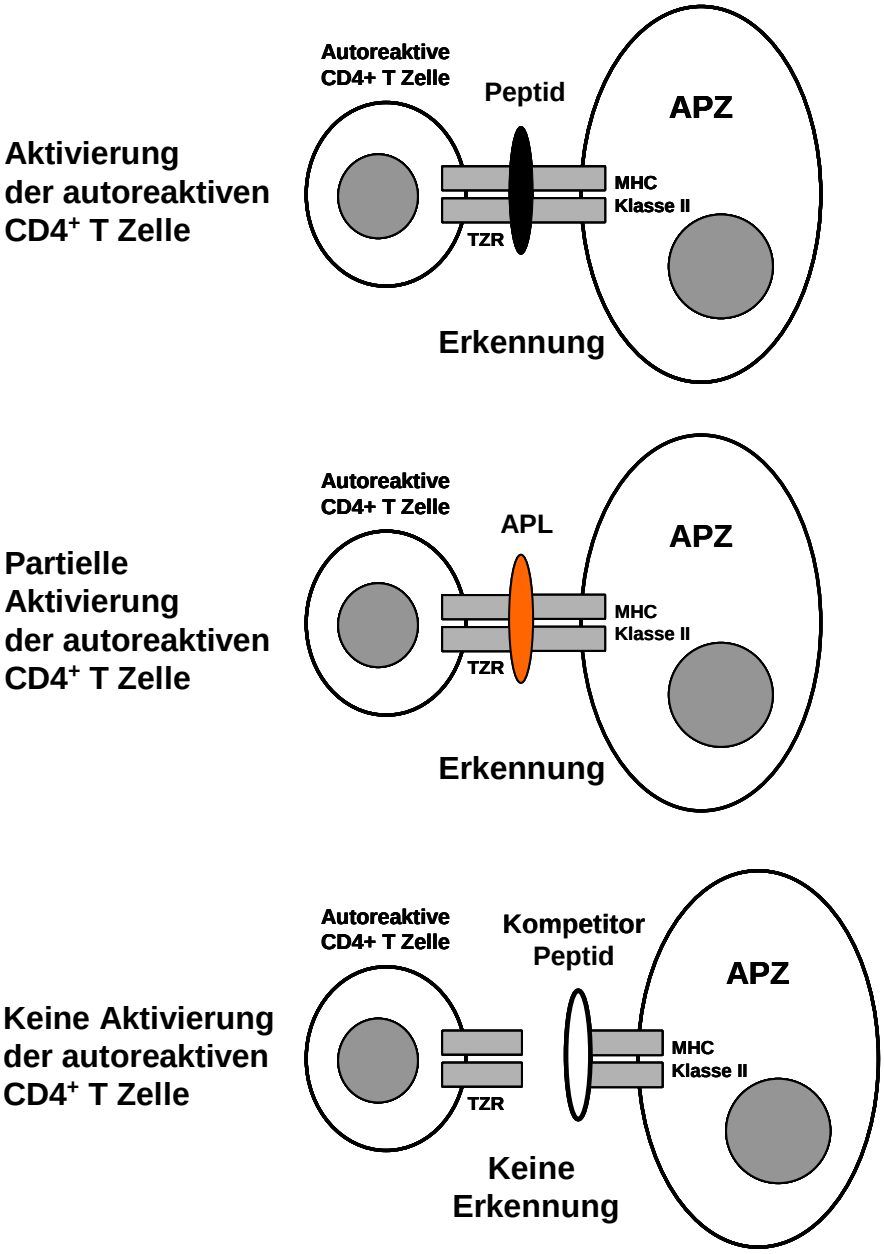

Bild 4. Schematische Darstellung der unterschiedlichen Wirkungsweisen von enzephalitogenem Peptid, APL und Kompetitorpeptid auf das Aktivierungsverhalten autoreaktiver T Zellen.

Publikation 7

Weissert, R., Kuhle, J., de Graaf, K.L., Wienhold, W., Herrmann, M.M., Müller, C., Forsthuber, T.G., Wiesmüller, K.H., and Melms, A. (2002). High immunogenicity of intracellular myelin oligodendrocyte glycoprotein epitopes. ***J Immunol. 169***, 548-556.

In der Studie wurden T Zellreaktivitätsprofile von MS Patienten und gesunden Kontrollen, die bezüglich des genetischen Risikofaktors HLA-DRB1*1501 stratifiziert worden waren, untersucht. Erstmalig konnte ein dominantes T Zell Epitop, MOG 146-154, innerhalb des intrazellulären Anteils von MOG eingegrenzt werden. Erstaunlicherweise konnte für die Kontrollpersonen ein breiteres Reaktionsprofil gegenüber MOG nachgewiesen werden als für die MS Patienten. Des weiteren erwiesen sich die transmembrane und intrazelluläre Domäne von MOG im Vergleich zum extrazellulären Proteinanteil als immunogener. Die Rolle von MOG als eine für die Immunpathogenese der MS potentiell relevante Zielstruktur konnte durch diese neuen Daten unterstützt werden.

Dieses ist die bisher umfassendste Studie bezüglich der Reaktivität gegenüber MOG in MS Patienten (Kerlero de Rosbo et al., 1993; Kerlero de Rosbo et al., 1997; Wallström et al., 1998; Lindert et al., 1999; Hellings et al., 2001; Minohara et al., 2001) (Tafel 9). Sehr überraschend war an diesen Ergebnissen der Befund, dass vor allem der intrazelluläre MOG Anteil beim Menschen immunogen ist. Die Ursache dafür könnte an MOG-ähnlichen Molekülen, welche in peripheren lymphoiden Organen, wie dem Thymus, exprimiert werden, liegen (Henry et al., 1999; Stefferl et al., 2000). Diese MOG ähnlichen Moleküle, wie die Butyrophillone, sind nur zum extrazellulären Anteil der MOG Sequenz homolog, jedoch nicht zum transmembranen und intrazellulären Anteil. Dieses könnte bedingen, dass T Zellen, welche den extrazellulären MOG Anteil erkennen, zu einem höheren Grade Toleranzmechanismen unterworfen sind im Vergleich zu T Zellen, welche gegen den transmembranösen oder intrazellulären Anteil gerichtet sind.

Die Daten unterstreichen, dass MOG als Autoantigen beim Menschen mit hoher Wahrscheinlichkeit eine Bedeutung hat. Sie legen nahe, dass die Immunogenität und vor allem Enzephalitogenität auch gegenüber dem transmembranen und inneren MOG Anteil in EAE Modellen genauer untersucht werden sollte.

Determinanten/Methodik	MHC Restriktion	Befund	Referenz
Kurzzeitkulturen (Proliferation) *T Zelllininien* MOG 1-22 MOG 34-56 MOG 64-96	Nicht bestimmt	Mehr Reaktivitäten bei MS Patienten im Vergleich zu Kontrollen. Keine HLA Stratifikation.	(Kerlero de Rosbo et al., 1997)
Kurzzeitkulturen (Elispot) MOG 38-60 MOG 63-87 MOG 76-100 MOG 89-113 MOG 162-182	Nicht bestimmt	Mehr Reaktivitäten bei MS Patienten im Vergleich zu Kontrollen. HLA stratifiziert.	(Wallström et al., 1998)
T Zelllinien MOG 1-26 MOG 14-39 MOG 27-50 MOG 38-60 MOG 76-100	 DR Nicht bestimmt DR DR DR	Leichte Unterschiede im Reaktivitätsprofil von MS Patienten und Kontrollen.	(Lindert et al., 1999)
Kurzzeitkulturen (Elispot)	Nicht bestimmt	Keine Unterschiede zwischen MS Patienten und Kontrollen.	(Hellings et al., 2001)
T Zelllinien 'Konventionelle' MS MOG 112-132 MOG 149-169 'Optikospinale' MS MOG 33-48 MOG 35-55 MOG 95-115 MOG 140-160 MOG 149-169 MOG 167-186 MOG 184-204 MOG 193-213	 DR, DQ DR, DQ DR DR DR DR DR Klasse II Klasse II DR	Mehr Reaktivitäten bei MS Patienten im Vergleich zu Kontrollen.	(Minohara et al., 2001)

Determinanten/Methodik	Restriktion	Individuen	Referenz
Kurzzeitkulturen (Elispot) MOG 81-96 MOG 125-140 MOG 129-144 MOG 133-148 MOG 141-145 MOG 145-160 MOG 169-184 MOG 173-188 MOG 181-196 MOG 189-204 MOG 201-216		Stratifiziert für DRB1*1501. Weniger Reaktivitäten bei MS Patienten im Vergleich zu Kontrollen.	(Weissert et al., 2002)
MOG 144-152 MOG 145-153 MOG 146-154	DR	MOG 146-154 dominante T Zelldeterminante.	
Humanisiertes Mausmodell	DRB1*0401	Transgene Mäuse	(Forsthuber et al., 2001)
Kurzzeitkulturen (Elispot) MOG 91-108 MOG 99-107			

Tafel 9. MOG Determinanten beim Menschen.

Zusammenfassung und Diskussion

Die vorliegende Arbeit ‚Experimentelle neuroimmunogenetische Untersuchungen zur Autoaggression und Therapie der Multiplen Sklerose' untersucht im Ratten- und im humanisierten transgenen Mausmodell die genetische Regulation der Autoaggression im ZNS im Zusammenspiel mit Myelinantigenen. Bei MS Kranken und Kontrollpersonen wurde die Immunogenität und Enzephalitogenität des Myelinantigens MOG analysiert. Des weiteren werden in der Arbeit zwei neue therapeutische Prinizipien zur Behandlung der MS eingeführt und genauer untersucht. Die Arbeit hat eine innere Kohärenz, weshalb der Weg der kumulativen Habilitationsschrift aus verschiedenen Einzelpublikationen beschritten wurde.

In der Arbeit wurde gezeigt:

- *Die selektive Expansion gewisser TZRBV Ketten bei der EAE und möglicherweise auch der MS ist durch die Kombination von MHC Klasse II Molekülen und der Präsentation von Selbst- versus Fremdantigen bedingt*. Diese Erkenntnis war neu. Sie hat zu einem verbesserten Verständnis der Beurteilung von T Zellexpansionen bei der EAE und der MS geführt. Die Daten unterstreichen den Einfluss von Umweltfaktoren wie viralen oder bakteriellen Infektionen auf das T Zellrepertoire und die Möglichkeit eines molekularen Mimikry beim Anstoß und der Aufrechterhaltung der MS (Wucherpfennig and Strominger, 1995).

- *Es wurde in ingezüchteten Rattenstämmen nach Immunisierung mit dem Myelinprotein MOG ein Tiermodell der MS etabliert, welches dieser um vieles ähnlicher ist als jedes andere bisher etablierte Tiermodell* (Wekerle et al., 1994). Im Vergleich zu Studien an Affen (Marmosetten und Makaken) bietet das Rattenmodell den Vorteil, dass eine große Anzahl genetisch identischer Nachkommen schnell gezüchtet werden kann und eine Vielzahl von ingezüchteten Stämmen zur Verfügung steht (Genain et al., 1995; Günther and Walter, 2001). Bei Mäusen wurde bisher nicht das gleiche umfangreiche Spektrum an klinischen Verläufen und histopathologischen Läsionen abhängig von MHC und nicht-MHC Genen induziert. Damit ist das Rattenmodell vom Ausmaß der Charakterisierung auf immunologischem, genetischem,

klinischem und neuropathologischem Gebiet den anderen MOG Modellen in Mäusen und Affen derzeit überlegen.

Mittels dieses MS-ähnlichen Tiermodells konnte gezeigt werden:

- *Der MHC Haplotypus bestimmt die Suszeptibilität, den Krankheitsverlauf und das Läsionsmuster im ZNS.* Es ist bereits lange bekannt, dass die Suszeptibilität der EAE durch den MHC beeinflusst wird (Williams and Moore, 1973; Gasser et al., 1973). Neu konnte gezeigt werden, dass der MHC Haplotypus den Krankheitsverlauf und das Läsionsmuster bestimmt.

- *Die MHC Klasse II Region stellt den Hauptsuszeptibilitätslokus dar, die MHC Klasse II vermittelten Effekte werden jedoch durch weitere der 120 MHC Gene moduliert.* Der Befund, dass die MHC Klasse II Region den Hauptsuszeptibilitätsfaktor in der EAE darstellt ist nicht neu oder überraschend, da diese einen fundamentalen Einfluss auf das Aktivierungsverhalten von T Zellen hat. In dem der MS sehr ähnlichen Tiermodell der MOG-EAE unterstreichen diese Daten, dass die MHC Klasse II Region mit hoher Wahrscheinlichkeit einen starken Einfluss auf die MS ausübt und die MS eine Autoimmunerkrankung ist. Neu wurde gezeigt, dass weitere MHC Gene einen Einfluss auf die Suszeptibilität nehmen. Dazu gehören die MHC Klasse I Gene und Gene telomer zu den MHC Klasse II Genen. Kürzlich wurde ein modulierender Einfluss von MHC Klasse I Genen auf MHC Klasse II vermittelte Suszeptibilitätseffekte bei der MS gezeigt (Fogdell-Hahn et al., 2000). Solche genetischen Einflüsse sollen in den nächsten Jahren genauer charakterisiert werden.

- *Die notwendige Menge der Antigenexposition zur Krankheitsinduktion unterscheidet sich für verschiedene MHC Haplotypen und korreliert zur Auslösung einer Immunantwort.* Dieser Befund ist neu und weist bei Übertragung auf den Menschen darauf hin, dass Individuen mit bestimmten Haplotypen bei Kontakt mit einem bestimmten Umweltfaktor (Antigen) ein hohes Risiko haben, eine Autoimmunerkrankung zu entwickeln.

- *Verschiedene Autoantigene wie unterschiedliche Myelinantigene können im Kontext mit unterschiedlichen MHC Haplotpyen Erkrankung auslösen.* Dieser

Befund wurde bisher nicht in der gleichen Klarheit und Eindeutigkeit gezeigt wie in unseren Studien zur MBP-EAE und MOG-EAE. Die Daten haben Bedeutung für Umweltantigene, die auf verschiedene MHC Haplotypen wirken. So findet sich bei Nordeuropäern und US Amerikanern eine Assoziation mit dem HLA-DR2 Haplotypus, in Sardinien jedoch mit dem HLA-DR4 Haplotypus (Marosu et al., 1988; Hillert and Olerup, 1993). Diese Befunde legen nahe, dass bei den unterschiedlichen Populationen verschiedene Umweltantigene zur Krankheitsauslösung und Aufrechterhaltung beitragen.

- *Nicht-MHC Gene können die MHC vermittelte Suszeptibilität unterdrücken.* Die Datenlage sprach zwar schon für diesen Befund (Happ et al., 1988). Erstmals wurde dieser Befund in dem MHC Haplotypus $RT1^{av1}$, welcher eine schubförmige und chronische Erkrankung begünstigt, erhoben. Die gewonnenen Daten waren Ausgangspunkt für eine Reihe von Kreuzungsexperimenten zwischen Rattenstämmen, die den gleichen MHC, jedoch unterschiedliche Hintergrundsgene tragen und sich in der Suszeptibilität unterscheiden. Es konnten bereits nicht-MHC Genregionen eingegrenzt werden, die einen Einfluss auf die Suszeptibilität in der MOG-EAE haben (Dahlman et al., 1999; Jagodic et al., 2001). Orthologe Vergleiche mit MS Patienten werden es möglicherweise erlauben, Suszeptibilitätsgene bei der MS mittels eines solchen Ansatzes einzugrenzen (Xu et al., 2001).

- *Die Determinante MOG 91-108 ist in verschiedenen Rattenstämmen (LEW.1AV1 [$RT1^{av1}$], DA [$RT1^{av1}$], COP [$RT1^{av1}$], PVG-$RT1^{a}$ [$RT1^{av1}$] und LEW.1N [$RT1^{n}$]) und in HLA-DR4 transgenen Mäusen enzephalitogen. Sie bindet mittelgut- bis gut an die restringierenden MHC Klasse II Moleküle.* Dieser Befund ist neu und legt nahe, dass auch beim Menschen die Immunantwort gegenüber MOG 91-108 enzephalitogen sein könnte. Die Daten zeigen in verschiedenen Haplotypen, dass die krankheitsauslösenden Peptide gut oder sehr gut an Autoimmunantwort- restringierende MHC Klasse II Moleküle binden.

- *Enzephalitogene Determinanten können bei der Analyse der peripheren Immunantwort mittels der derzeit verfügbaren Methoden unerkannt bleiben.*

Bisher wurde davon ausgegangen, dass in den EAE Modellen enzephalitogene Determinanten im peripheren Immunsystem nach Immunisierung mit enzephalitogenem Protein oder Peptid eine gut messbare T Zellantwort induzieren. Der Befund legt nahe, dass in der Peripherie und im ZNS unterschiedliche Ebenen der Toleranz existieren können. Dieses wird durch den MHC Haplotypus bestimmt. Es ist nun eine wichtige Aufgabe für die nächsten Jahre, die Regulation der MOG 91-108 spezifischen Immunantwort in der Peripherie und im ZNS genauer herauszuarbeiten.

- *MHC Klasse II Haplotypen unterscheiden sich in ihrem Bindungsverhalten gegenüber MOG Peptiden. Das Bindungsverhalten korrelierte mit der Heftigkeit und Schwere der Erkrankung*. Dieser Befund ist neu und legt nahe, dass ein breiteres Bindungsverhalten von autoantigenen Peptiden auch zu einer vermehrten Präsentation von solchen Determinanten gegenüber T Zellen führen kann, was wiederum Einfluss auf die Heftigkeit, die Schwere und den Verlauf der Erkrankung haben kann.

- *Es wurde das Peptidbindungsmuster für das RT1.D^{n} Molekül der Ratte beschrieben.* Dieses ist das erste Bindungsmuster für ein RT1.D Molekül von Ratten. Bisher ist nur das RT1.B^{l} Bindungsmuster und Ligandenmotiv bekannt (Reizis et al., 1996; Wauben et al., 1997).

Bei MS Patienten und Probanden konnte gezeigt werden:

- *Transmembranes und intrazelluläres MOG ist sehr immunogen beim Menschen.* Wir zeigen hier erstmals, dass der transmembrane und intrazelluläre Anteil von MOG sehr immunogen ist. Das enzephalitogene Potential dieser Sequenzen muss nun genauer untersucht werden.

- *Im Vergleich zu MS Kranken zeigen Kontrollpersonen mehr T Zellreaktivitäten in der Peripherie gegenüber MOG Peptiden.* Dieser Befund war unerwartet und spricht gegen die derzeitige Lehrmeinung, dass bei MS Patienten höhere T Zellreaktivitäten gegenüber Myelinproteinen vorhanden sein sollten. Der Befund würde möglicherweise darauf hindeuten, dass T Zellen im Zielorgan konsumiert werden und nicht mehr in der Peripherie zur Verfügung stehen (Flügel et al., 2001). Alternativ könnte eine Veränderung regulatorischer

Mechanismen bei MS Patienten vorliegen, welche zur Reduktion krankheitssupprimierender Reaktivitäten führen.

- *Bei MS Patienten und auch Kontrollpersonen findet sich eine dominante T Zellantwort gegenüber der intrazellulären MOG Determinante MOG 146-154.* Erstmals wurde eine dominante T Zelldeterminante bei MOG 146-154 eingegrenzt.

Therapeutisch konnte gezeigt werden:

- *Peptide, die hoch-affin an Autoimmunitäts-assoziierte MHC Klasse II Moleküle binden und keine Sequenzhomologien zu bekannten Proteinen haben, können die EAE unterdrücken.* Der gewählte Ansatz des rationalen Designs hoch-affin bindender Peptide mittels kombinatorischer Peptidbibliotheken ist neu. Zudem konnte erstmals die fulminante MOG-EAE in der LEW.1N Ratte unterdrückt werden. Darüber hinaus stützen die Untersuchungen den fundamentalen Einfluss der MHC Klasse II Region auf die MOG-EAE. Im Vergleich zu APL, die nur gewisse T Zellpopulationen modulieren, bietet die Blockade Autoimmunitäts-assoziierter MHC Moleküle eine verbesserte Option Krankheit zu unterdrücken. Zudem haben die hoch-affinen Peptide eine starke immunogene Wirkung, die per se durch Beeinflussung des T Zellrepertoires auch eine Modulation des autoimmunen T Zellrepertoires bedingen könnte.

- *DNS Vakzine für autoantigene Sequenzen haben eine hohe Spezifität und können die EAE unterdrücken.* Diese Arbeit ist eine Fortführung unserer grundlegenden Arbeiten zur Vakzinierung mit nackter DNS in der EAE (Lobell et al., 1998; 1999). Erstmals wurde gezeigt, dass der Effekt der Vakzinierung mit autoantigenen Sequenzen als nackte DNS relativ spezifisch ist und keine *bystander* Supprimierung hervorgerufen wird.

Damit wurden in der Arbeit eine Reihe von grundlegenden neuen Erkenntnissen gewonnen, die für die experimentelle und klinische Neuroimmunologie und Neurologie wichtig sind. Diese Erkenntnisse führen zu einem verbesserten Verständnis der Immungenetik der Multiplen Sklerose anhand der experimentellen Studien in der EAE und eröffnen neue Wege für die Therapie. Sie bilden die Grundlage für weiterführende Untersuchungen in der EAE und beim MS Patienten.

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Publikation 1

Molecular and Genetic Requirements for Preferential Recruitment of TCRBV8S2$^+$ T Cells in Lewis Rat Experimental Autoimmune Encephalomyelitis[1]

Robert Weissert,[2]* Anders Svenningsson,* Anna Lobell,*† Katrien L. de Graaf,* Roland Andersson,† and Tomas Olsson*

The underlying mechanisms behind the preferential expression of select TCRBV products in certain autoimmune illnesses, such as multiple sclerosis and some models of experimental autoimmune encephalomyelitis (EAE), have principally remained enigmatic. In this study, we examined the mutual role of nonself- vs self-origin of antigenic myelin basic protein (MBP) peptides and given MHC haplotypes in relation to the relative frequency of activated TCRBV8S2$^+$ T lymphocytes in the Lewis (LEW) rat EAE model. Inbred MHC (RT1) congenic LEW rats (LEW (RT1^l), LEW.1AV1 (RT1^{av1}), and LEW.1W (RT1^u)) were immunized with the 63 to 88 peptide of the guinea pig MBP (MBP$_{GP}$63-88). Additionally, LEW rats were immunized with the corresponding autologous rat sequence (MBP$_{RAT}$63-88). Although EAE ensued in all MBP peptide/LEW rat strain combinations, only LEW rats immunized with the heterologous MBP$_{GP}$63-88 peptide elicited T cell responses encompassing a bias toward TCRBV8S2 expression, as determined by flow cytometric analyses. Reduction of TCRBV8S2$^+$ T cells led to mitigation of disease severity in LEW rats immunized with MBP$_{GP}$63-88, but not with MBP$_{RAT}$63-88, indicating that critical encephalitogenic characteristics are associated with this T cell subset. We conclude that the preferential recruitment of TCRBV8S2$^+$ T cells in the LEW rat EAE model is due to selective, high-avidity recognition of the nonself-MBP$_{GP}$63-88 in the context of the RT1.B^l molecule. This inference lends support to the notion that the highly restricted TCR repertoire of the self-MBP-reactive T cells in certain genetically predisposed multiple sclerosis patients may have its source in a multistep molecular mimicry event. ***The Journal of Immunology,*** **1998, 160: 681–690.**

Experimental autoimmune encephalomyelitis (EAE)[3] is a central nervous system (CNS) disorder with clinical and pathologic characteristics similar to those of the human demyelinating CNS disease multiple sclerosis (MS). Autoaggressive T cells directed against determinants of discrete CNS-associated myelin proteins play a critical role in the pathogenesis of EAE (1). To date, most studies have focused on myelin basic protein (MBP) as constituting an abundant self-Ag of CNS. In various animal species/strains, a single or a few immunodominant determinants of MBP are responsible for induction of EAE. Besides, in certain EAE models, specific recognition of such MBP determinants recruits encephalitogenic T cells that display a restricted TCRBV expression (2–6). Similar observations have been reported on MS (7–9), while other reports are negative in this respect (10, 11). Lewis (LEW) rat-derived T cells specific for the dominant encephalitogenic 68 to 88 sequence of the GP MBP molecule, MBP$_{GP}$68-88, are CD4$^+$CD8$^-$ or CD4$^-$CD8$^-$ (12, 13) and TCR(A/B)$^+$, among which the TCRBV8S2 product is prevalently overexpressed. Interestingly, there is preferential TCRAV chain usage as well in this particular LEW rat model (14). However, LEW rats do not elicit a restricted TCRBV pattern in response to the second, dominant encephalitogenic sequence, MBP89-101, conserved in rat, mouse, and guinea pig (GP) MBP (15). Moreover, following immunizations with full-length MBP and MBP-derived peptides, other strains of rats do not generate preferential activation of TCRBV8S2$^+$ T lymphocytes (16).

The molecular mechanisms behind the skewed usage of particular TCRBV products are largely unknown. Components of the trimolecular complex, the TCR, the MHC molecule, and the antigenic peptide, are obvious candidates for such influences and, consequently, the subjects of interest in this study (17, 18). The importance of MHC molecules is also nourished by other observations. Although susceptibility/resistance for both MS and EAE is polygenically controlled (19, 20), the strongest association described is with alleles within the MHC. For instance, the HLA-Dw2 haplotype is a decisive susceptibility gene region in MS (21). Circumstantial evidence strongly suggests that the impact of allele-specific MHC molecules on induction and maintenance of autoimmune diseases is related to their ability to selectively present antigenic peptides to T cells, both in the thymus and in the periphery (22).

The aims of the present study of LEW rat EAE were twofold: firstly, to evaluate the dependence of TCRBV8S2 usage by encephalitogenic T cells on MHC allele-specific products in conjunction with autologous and heterologous MBP63-88 peptides, and, secondly,

*Neuroimmunology Unit, Center of Molecular Medicine, Karolinska Hospital, Stockholm, Sweden; and †Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden

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[2] Address correspondence and reprint requests to Dr. Robert Weissert, Neuroimmunology Unit, Center of Molecular Medicine L8:04, Karolinska Hospital, S-17176 Stockholm, Sweden. E-mail address: robert.weissert@cmm.ki.se

[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; CNS, central nervous system; MS, multiple sclerosis; MBP, myelin basic protein; LEW, Lewis; GP, guinea pig; LN, lymph node; MNC, mononuclear cell; p.i., post-immunization; IC_{50}, 50% inhibitory concentration; PE, phycoerythrin; CM, complete medium; FL, fluorescence channel; T_{79}, threonine at position 79; S_{79}, serine at position 79; ELISPOT, enzyme-linked immunospot.

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Table I. *Inbred rat strain designations and RT1 haplotypes*

Strain	Haplotype	Class I RT1.A	Class II RT1.B	Class II RT1.D	Class III	Class I RT1.C
LEW	l	l	l	l	l	l
LEW.1AV1 (DA)[a]	av1	a	a	a	av1	av1
LEW.1W (WP)	u	u	u	u	u	u

[a] Donor strains are indicated within parentheses.

to quantify the preferential recruitment of TCRBV8S2$^+$ T cells. Inbred MHC congenic LEW rats (LEW (RT1^l), LEW.1AV1 (RT1^{av1}), and LEW.1W (RT1^u)) were selected for studies on the basis of previously demonstrated MHC restriction patterns (23, 24). EAE was induced in all LEW rat strains immunized with the 63 to 88 peptide of the GP MBP (MBP_{GP}63-88) and, in addition, in LEW rats immunized with the corresponding autologous rat MBP peptide (MBP_{RAT}63-88). Quantification of activated TCRBV8S2$^+$ T cells from blood, draining lymph node (LN), and CNS was examined, as measured by FACS analyses. Only LEW rats immunized with the heterologous MBP_{GP}63-88 sequence displayed T cell responses characterized by preferential TCRBV8S2 expression. It was herein demonstrated that the differential usage of TCRBV8S2 in the congenic LEW rat strains is determined by the relationship between allele-specific MHC class II products and the actual sequence of the encephalitogenic MBP peptide.

Materials and Methods

Rats

Male rats (Table 1), 8 to 12 wk of age, were used in all experiments. LEW (RT1), LEW.1AV1 (RT1^{av1}), and LEW.1W (RT1^u) rats were originally obtained from the Zentralinstitut für Versuchstierzucht, Hannover, Germany (25). Subsequently, they were locally bred in filter boxes and routinely tested for specific pathogens. Breeding pairs were checked for homozygosity by examination of a microsatellite marker located within the RT1 region.

Synthetic peptides

MBP_{GP}63-88 (AARTTHYGSLPQKSQRSQDENPVVHF), MBP_{RAT}63-88 (HTRTTHYGSLPQKSQRTQDENPVVHF), and $MBP_{RAT/GP}$89-101 (VHFFKNIVTPRTP) sequences were synthesized by F-moc/HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate) strategy (Dr. A. Engström, Department of Medical and Physiologic Chemistry, University of Uppsala, Sweden). Peptides were purified by reversed-phase chromatography and, subsequently, analyzed by plasma desorption mass spectroscopy. The degree of purity of the used peptides was >99%. The N-terminally biotinylated peptide MBP_{GP}72-85 (LPQKSQRSQ DENPV) was a generous gift from Dr. G. Jung, Institute of Organic Chemistry, University of Tübingen, Germany.

mAbs and reagents

All mAbs used for FACS analysis were purchased from PharMingen (San Diego, CA): FITC-labeled anti-rat TCR(A/B) (clone R73, mouse IgG1,κ), phycoerythrin (PE)-labeled anti-rat TCRBV8S2 (clone R78, mouse IgG1,κ), PE-labeled anti-rat TCRBV10 (clone G101, mouse IgG2a,κ), FITC-labeled anti-rat CD25 (IL-2R α-chain, clone WT.1, mouse IgG2a,κ), FITC-labeled mouse IgG1,κ (clone 107, 3), and PE-labeled mouse IgG2a,κ (clone G155-178). The anti-rat TCRBV8S2 mAb (clone R78, mouse IgG1,κ) was also used for in vivo-depletion studies. The hybridoma MRC-OX-6 (European Collection of Cell Cultures, Salisbury, U.K.), secreting a mouse anti-rat RT1.B-specific Ab (IgG1), was cultured in DMEM (Life Technologies, Paisley, Scotland) supplemented with 10% FCS (Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 1% glutamine (Life Technologies). The OX-6 Abs were purified on a protein G column (Pharmacia, Uppsala, Sweden). Con A was purchased from Sigma (St. Louis, MO).

Induction and evaluation of EAE

For induction of EAE, rats were injected intradermally at the base of the tail with a total volume of 200 μl of inoculum containing 200 μg of either MBP_{GP}63-88 or MBP_{RAT}63-88 in saline mixed (1:1) with CFA, which consisted of IFA (Sigma) and 1 mg of heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco Laboratories, Detroit, MI). Immunizations were performed under inhalation anesthesia with methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL).

Animals were clinically scored and weighed on a daily basis up to 25 days post-immunization (p.i.). Symptoms were scored as follows: grade 1, tail weakness or tail paralysis; grade 2, hind leg paraparesis; grade 3, hind leg paralysis; grade 4, complete paralysis (tetraplegy), moribund state, or death.

Fractionation and cultivation of mononuclear cells (MNCs) from LN, spleen, and blood

Under deep anesthesia, draining inguinal LN were dissected out and put in DMEM. MNCs were isolated by careful disruption of the LN; washed twice in DMEM; resuspended in complete medium (CM) containing DMEM supplemented with 1% rat serum, 1% penicillin/streptomycin, 1% glutamine, and 50 μM 2-ME (Life Technologies); and flushed through a 70-μm plastic strainer (Falcon; Becton Dickinson, Mountain View, CA). MNC from spleen were prepared in the same way as for LN with the difference that RBC were lysed with lysing buffer, consisting of 0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM Na_2 EDTA adjusted to pH 7.4.

MNC were cultured at a concentration of 2×10^6 cells/ml in 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark) with 100 μl of cell suspension per well at 37°C in a humidified atmosphere containing 5% CO_2. For each rat/Ag combination, MNC were cultured for 72 h, washed twice in DMEM, adjusted to a concentration of 20×10^6 cells/ml, and immunostained for FACS analysis.

Blood was drawn by heart puncture under deep anesthesia and collected in heparinized blood containers (Becton Dickinson). Blood MNC were then isolated on Lymphoprep density gradients (Nygaard, Oslo, Norway), washed twice in DMEM, and resuspended in CM at a concentration of 20×10^6 cells/ml for FACS analysis.

Recovery of MNC from CNS

Deeply anesthetized animals were perfused with 75 ml of PBS. CNS was carefully dissected out and transferred to a 50-ml centrifugation tube (Falcon) containing 35 ml of DMEM, gently agitated to release cells into the medium, and floated through a 70-μm plastic strainer (Falcon). Contaminating RBC were removed through Lymphoprep density gradient centrifugation, and MNC were collected from the interphase, washed twice in DMEM, and resuspended in CM for immunostaining.

Proliferation assay

All proliferative experiments were performed in triplicates in 96-well round-bottom microtiter plates. A total of 2×10^5 MNC/well were cultivated in CM with or without the relevant Ags for 60 h and, subsequently, pulsed with 0.5 mCi of [^{3}H]TdR (Amersham, Buckinghamshire, U.K.) per well for an additional 12 h. DNA was collected on glass fiber filters (Skatron, Sterling, VA) and [^{3}H]TdR incorporation was measured in a beta counter (Beckman, Palo Alto, CA).

Enumeration of cells secreting Ag-specific IFN-γ

To enumerate T cells secreting IFN-γ after Ag exposure, the ELISPOT method was used (23, 24). Nitrocellulose-bottomed 96-well plates (MAHA; Millipore, Molsheim, France) were coated with the mAb DB1 (a

generous gift of Dr. Peter van der Meide, TNO Primate Centre, Rijswijk, the Netherlands), which reacts with rat IFN-γ. Following washing with PBS, the plates were blocked with DMEM containing 5% FCS (Life Technologies). A total of 4×10^5 cells per well in 200 μl of CM were added to the plates and incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. For each Ag, triplicate determinations were performed. Afterward cells were discarded and plates were washed four times with PBS. Secreted and bound IFN-γ was visualized with biotinylated DB12 (also a generous gift of Dr. Peter van der Meide), which has another binding site on IFN-γ than DB1, avidin-biotin peroxidase (Vector Laboratories, Burlingame, CA) and, subsequently, by staining with carbazole (Sigma).

Immunostaining and flow cytometry

For flow cytometric analysis, cells isolated from blood, LN, and CNS as well as cultured cells were immunostained in 96-well microtiter plates. Predetermined optimal concentrations of mAbs were added to each cell suspension (50 μl). Plates were then incubated in the dark on ice, washed twice in PBS, and resuspended in 200 μl of PBS containing 1% paraformaldehyde. Finally, samples were analyzed by use of a Becton Dickinson FACSort flow cytometer.

To obtain large enough numbers of relevant $TCRBV^+$ T cells for reliable analysis of CD25 co-expression, specific acquisition gates were used. This procedure allowed saving of events signifying lymphocytes expressing either TCRBV8S2 or TCRBV10 products, as defined by light scatter properties in combination with positive signals visualized in the second fluorescence channel (FL2). By this approach, 2000 to 5000 cells per sample of each specific $TCRBV^+$ T cell subset could be analyzed for CD25 co-expression in the first fluorescence channel (FL1). In general, CNS samples contained large amounts of cellular debris that frequently co-localized with double-stained T lymphocytes visualized in the regular flow cytometry plots. To sort out irrelevant events (debris), the third fluorescence channel (FL3) was utilized (26). In the FL1 vs FL3 plot, $TCR(A/B)^+$ T cells could be gated and these events were then further analyzed for expression of specific TCRBV products in an FL1 vs FL2 plot.

Frequencies of the relevant $TCRBV^+$ T cell subsets are represented as percentages of the total number of $TCR(A/B)^+$ T cells. In most T cell samples, the relevant TCRBVs and CD25 were co-expressed at a level equal to that of the negative controls. Hence, to define $CD25^+$ cells, the fluorescence intensity-level marker was set directly above the negative population. The same marker position was consistently used for both $TCRBV^+$ T cell subsets.

Depletion of TCRBV8S2$^+$ T cells

For depletion studies, a total volume of 200 μl containing 150 μg of the R78 mAb in PBS was injected i.p. in each rat on day 8 p.i. The degree of depletion of $TCRBV8S2^+$ T cells was tested on day 9 p.i. by FACS analysis of blood cells from each treated rat.

Purification of RT1.B^l molecules

$RT1.B^l$ molecules were purified from LEW rat LN, thymic, and splenic tissues by affinity chromatography using the OX-6 mAb (anti-RT1.B) coupled to CNBr-activated Sepharose-4B (Pharmacia) (27). Purity of the eluted proteins was assessed by SDS-PAGE and subsequent silver staining.

Peptide binding assay

For the peptide binding affinity assay, biotinylated $MBP_{GP}72$-85 was used as the reference peptide. Briefly, $RT1.B^l$ molecules (200 nM) were incubated with the reference peptide (500 nM) in the presence of various concentrations of each of the unlabeled relevant peptides, $MBP_{GP}63$-88, $MBP_{RAT}63$-88, and $MBP_{GP/RAT}89$-101. The binding buffer consisted of 2 mM EDTA, 25 mM Na_2CO_3, 50 mM Tris/HCL, 0.01% azide, 0.1 mM PMSF, and 0.1% Nonidet P-40 (Boehringer, Mannheim, Germany), titrated to pH 5 with a citrate solution. After 72 h of incubation at room temperature, the peptide-MHC complexes were quantified by ELISA (28). The signal intensity of the enzymatic reaction was directly proportional to the concentration of the newly formed peptide-MHC complexes under the conditions used. The relative binding affinity of the relevant peptide is expressed as the peptide concentration needed for 50% inhibition of the binding of the reference peptide (IC_{50}). IC_{50} values were determined in three separate experiments.

Statistical analysis

Student's *t* test was used for normally distributed variables. When the data did not fulfill the criteria of being normally distributed, nonparametric statistics (Mann-Whitney *U* test) was used.

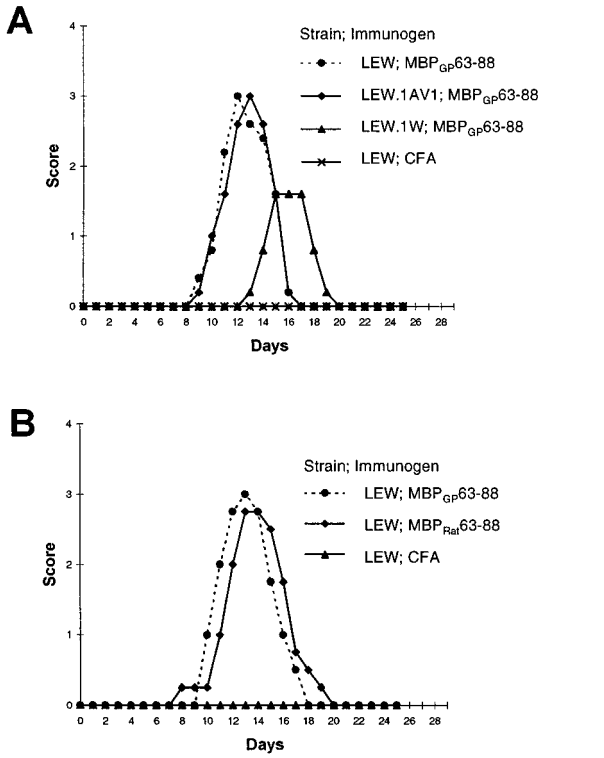

FIGURE 1. EAE-profile in relation to MHC haplotype and MBP peptide. Clinical EAE disease courses of $MBP_{GP}63$-88-primed LEW (n = 5), LEW.1AV1 (n = 5), and LEW.1W (n = 5) rats and CFA-injected controls (n = 4), and of $MBP_{GP}63$-88-primed LEW rats (n = 4), $MBP_{RAT}63$-88-primed LEW rats (n = 4), and CFA-injected controls (n = 4) are illustrated in *A* and *B*, respectively. Rats were injected with 200 μg of $MBP_{GP}63$-88 or $MBP_{RAT}63$-88 peptides emulsified in CFA, followed for 25 days, and scored on a daily basis.

Results

Clinical EAE profile

RT1 congenic LEW rats immunized with highly purified MBP peptides, $MBP_{GP}63$-88 or $MBP_{RAT}63$-88, were monitored for EAE disease courses over a period of 25 days (Fig. 1). Following immunization with $MBP_{GP}63$-88, LEW and LEW.1AV1 rats displayed initial overt clinical signs on average at day 10 p.i. (Fig. 1*A*). Both rat strains generated a self-limiting monophasic disease profile, reaching a mean maximum disease score of grade 3. The semiresistant LEW.1W rat strain also developed a monophasic disease profile, but with a later onset (day 14 p.i.) and a lower mean maximum disease score (grade 1.6). Moreover, LEW rats immunized with $MBP_{RAT}63$-88 elicited a clinical course similar to that obtained from immunization with $MBP_{GP}63$-88 (Fig. 1*B*). None of the CFA-injected controls exhibited any signs of EAE.

Frequencies of activated peripheral TCRBV8S2$^+$ T lymphocytes

Next, we determined the relationship between the specific set of expressed MHC allelic products and the relative number of activated $TCRBV8S2^+$ T cells in MBP peptide-induced EAE. RT1 congenic LEW, LEW.1AV1, and LEW.1W rats were immunized with $MBP_{GP}63$-88 and, in addition, LEW rats with $MBP_{RAT}63$-88. Because of the known bias toward preferential TCRBV8S2 usage in LEW rat EAE, the activation state within the respective $TCRBV8S2^+$ T cell subset was analyzed for CD25 co-expression on day 12 p.i. Since $TCRBV10^+$ T cells have never been reported

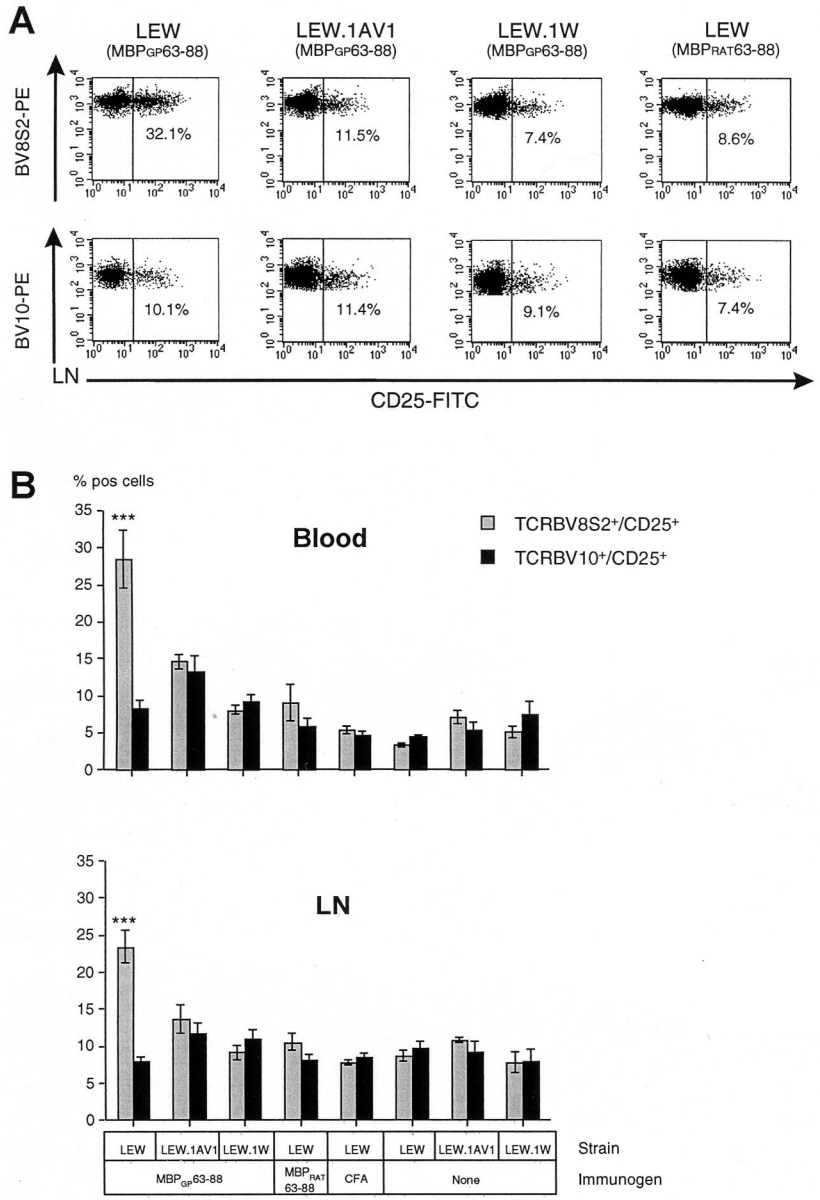

FIGURE 2. Frequencies of activated peripheral TCRBV8S2$^+$ T cells from MBP63-88-primed RT1 congenic LEW rat strains. *A* illustrates typical two-color flow cytometric analyses of TCRBV8S2$^+$/CD25$^+$ and TCRBV10$^+$/CD25$^+$ T cells from draining LN of a randomly chosen individual from the groups of RT1 congenic LEW rats immunized with MBP_{GP}63-88 and from LEW rats immunized with MBP_{RAT}63-88, mentioned in *B*. *B* depicts the frequencies of TCRBV8S2$^+$/CD25$^+$ and TCRBV10$^+$/CD25$^+$ T cells from blood and draining LN of MBP_{GP}63-88-primed LEW ($n = 7$), LEW.1AV1 ($n = 5$), and LEW.1W ($n = 4$) rats as well as MBP_{RAT}63-88-primed LEW rats ($n = 5$), as determined by two-color FACS analyses. CFA-injected LEW ($n = 4$) and naive LEW ($n = 4$), LEW.1AV1 ($n = 4$) and LEW.1W ($n = 4$) rats were included as controls. Blood and LN cells were obtained on day 12 p.i. Collection of cells, staining procedures, and evaluation of data were performed as described in *Materials and Methods*. Each bar indicates mean ± SEM. *** indicates statistical significance ($p < 0.0003$).

to be expanded or retracted during anti-MBP responses in the rat, each tested cell population was double stained in parallel with anti-TCRBV10 and anti-CD25 mAbs as control.

Within both the PBL- and the LN-derived T cell pool from MBP_{GP}63-88-primed LEW rats, the number of TCRBV8S2$^+$ T cells co-expressing CD25 was significantly increased ($p < 0.0003$), whereas the frequency of TCRBV10$^+$/CD25$^+$ T cells was unaffected (Fig. 2). Such a selective, increased expression of CD25 in the TCRBV8S2$^+$ T cell subset was not observed in LEW rats immunized with MBP_{RAT}63-88. Likewise, MBP_{GP}63-88-primed LEW.1AV1 and LEW.1W rats, CFA-injected, and naive controls exhibited unchanged frequencies of both the TCRBV8S2$^+$/CD25$^+$ and the TCRBV10$^+$/CD25$^+$ T cell subset. Due to the delayed onset of clinical signs in the LEW.1W strain (Fig. 1*A*), we assessed CD25 expression on TCRBV8S2$^+$ T cells also on day 16 p.i. No preferential activation was observed (data not shown).

Expansions of peripheral TCRBV8S2$^+$ T lymphocytes

We also examined whether the specific activation of TCRBV8S2$^+$ T cells in LEW rats immunized with MBP_{GP}63-88 caused measurable expansions. On day 12 p.i., a significant, albeit modest expansion of TCRBV8S2$^+$ T cells was demonstrated in PBL from the LEW rat immunized with MBP_{GP}63-88 ($p < 0.05$) (Fig. 3, *A* and *B*), but not in the draining LN-derived T cell population (Fig. 3*B*). Importantly, neither LEW rats immunized with the autologous MBP_{RAT}63-88 peptide nor LEW.1AV1 and LEW.1W rats immunized with MBP_{GP}63-88 exhibited preferential expansions of TCRBV8S2$^+$ T cells. The frequencies of TCRBV10$^+$ T cells in immunized animals were indistinguishable from those observed in CFA-injected and naive controls. As expected, LEW.1W rats immunized with MBP_{GP}63-88, analyzed on day 16 p.i., did not display expansions within either of the TCRBV8S2$^+$ and TCRBV10$^+$ T cell subsets (data not shown).

Frequencies of CNS-infiltrating TCRBV8S2$^+$ T lymphocytes

In view of the over-representation of activated TCRBV8S2$^+$ T cells in blood from MBP_{GP}63-88-primed LEW rats, we investigated the degree of passage through the blood-brain barrier into the CNS compartment. The relative presence of TCRBV8S2$^+$ T cells was thus determined at the site at which the autoantigenic MBP target molecule is exposed. LEW, LEW.1AV1, and LEW.1W rats

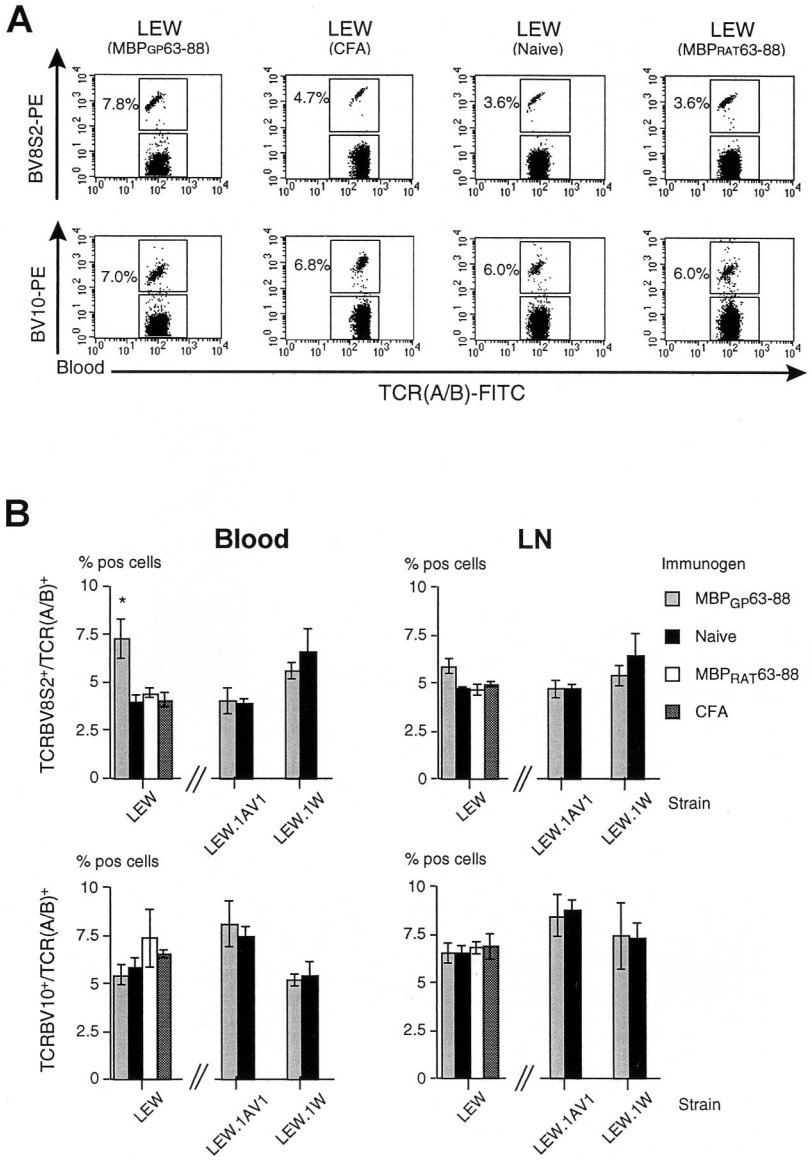

FIGURE 3. Expansions of peripheral TCRBV8S2$^+$ T lymphocytes in MBP$_{GP}$63-88-primed LEW rats. *A* illustrates typical two-color flow cytometric analyses of TCRBV8S2$^+$/TCR(A/B)$^+$ and TCRBV10$^+$/TCR(A/B)$^+$ T cells from the blood of a randomly chosen individual from the groups of LEW rats immunized with either MBP$_{GP}$63-88, MBP$_{RAT}$63-88, or CFA alone and from a naive animal, mentioned in *B*. *B* indicates the frequencies of TCRBV8S2$^+$/TCR(A/B)$^+$ and TCRBV10$^+$/TCR(A/B)$^+$ T cells from blood and draining LN of LEW ($n = 7$), LEW.1AV1 ($n = 5$), and LEW.1W ($n = 4$) rats immunized with MBP$_{GP}$63-88 and LEW rats ($n = 5$) immunized with MBP$_{RAT}$63-88, as determined by two-color FACS analyses. CFA-injected LEW ($n = 4$) and naive LEW ($n = 4$), LEW.1AV1 ($n = 4$), and LEW.1W ($n = 4$) rats were included as controls. Blood and LN cells were obtained on day 12 p.i. Collection of cells, staining procedures, and evaluation of data were performed as described in *Materials and Methods*. Each bar represents the mean ± SEM. * indicates statistical significance ($p < 0.05$).

were immunized with MBP$_{GP}$63-88 or MBP$_{RAT}$63-88, and CNS-infiltrating cells were recovered on day 12 p.i. Almost 30% of T lymphocytes from the CNS of LEW rats immunized with MBP$_{GP}$63-88 expressed TCRBV8S2 ($p < 0.0003$) (Fig. 4). This relative frequency of CNS-associated TCRBV8S2$^+$ T cells was markedly higher than that recorded in LEW rat T cell populations derived from blood ($p < 0.0003$) and LN ($p < 0.0003$). Importantly, no increased frequency of TCRBV8S2$^+$ T lymphocytes was recorded in LEW rats immunized with the autologous MBP$_{RAT}$63-88 peptide, or LEW.1AV1 and LEW.1W rats immunized with MBP$_{GP}$63-88. MNC recovered from LEW.1W rats on day 16 p.i. showed normal frequencies of TCRBV8S2$^+$ and TCRBV10$^+$ T cells (data not shown).

T lymphocyte cross-reactivity with MBP$_{GP}$63-88 and MBP$_{RAT}$63-88 in differentially primed LEW rats

Since LEW rats had shown marked differences after immunization with either MBP$_{GP}$63-88 or MBP$_{RAT}$63-88 in the preferential activation and recruitment of TCRBV8S2$^+$ T cells in vivo, we also tested for cross-reactivity with both peptides in vitro. Cells from draining LN of LEW rats immunized either with MBP$_{GP}$63-88 or MBP$_{RAT}$63-88 were isolated on day 12 p.i. and cultured with the MBP63-88 peptides, Con A, or medium alone. Irrespective of choice of immunogen, over 80% of Con A-stimulated TCRBV8S2$^+$ and TCRBV10$^+$ T cells co-expressed CD25 (data not shown). After immunization with MBP$_{GP}$63-88 and in vitro restimulation with the same peptide, the number of TCRBV8S2$^+$ T cells co-expressing CD25 was selectively increased as opposed to that of the TCRBV10$^+$/CD25$^+$ T cell subset ($p < 0.01$). Interestingly, this was also true, but to a lower extent, after culture with MBP$_{RAT}$63-88 ($p < 0.05$) (Fig. 5*A*). Thus, TCRBV8S2$^+$ T cells preferentially expanded due to the heterologous MBP$_{GP}$63-88 peptide, which could recognize and maintain an increased CD25 expression in response to the syngeneic MBP$_{RAT}$63-88 peptide. No significant changes in expression of CD25 on TCRBV8S2$^+$ cells were recorded after secondary stimulation in vitro with MBP$_{RAT}$63-88 and MBP$_{GP}$63-88 of cells from MBP$_{RAT}$63-88-primed LEW rats (Fig. 5*B*).

T cells from MBP$_{GP}$63-88-primed LEW rats displayed a proliferative response both to MBP$_{GP}$63-88 and MBP$_{RAT}$63-88, with the former eliciting a slightly higher response (Fig. 6*A*). T cells from MBP$_{RAT}$63-88-primed LEW rats proliferated roughly at similar

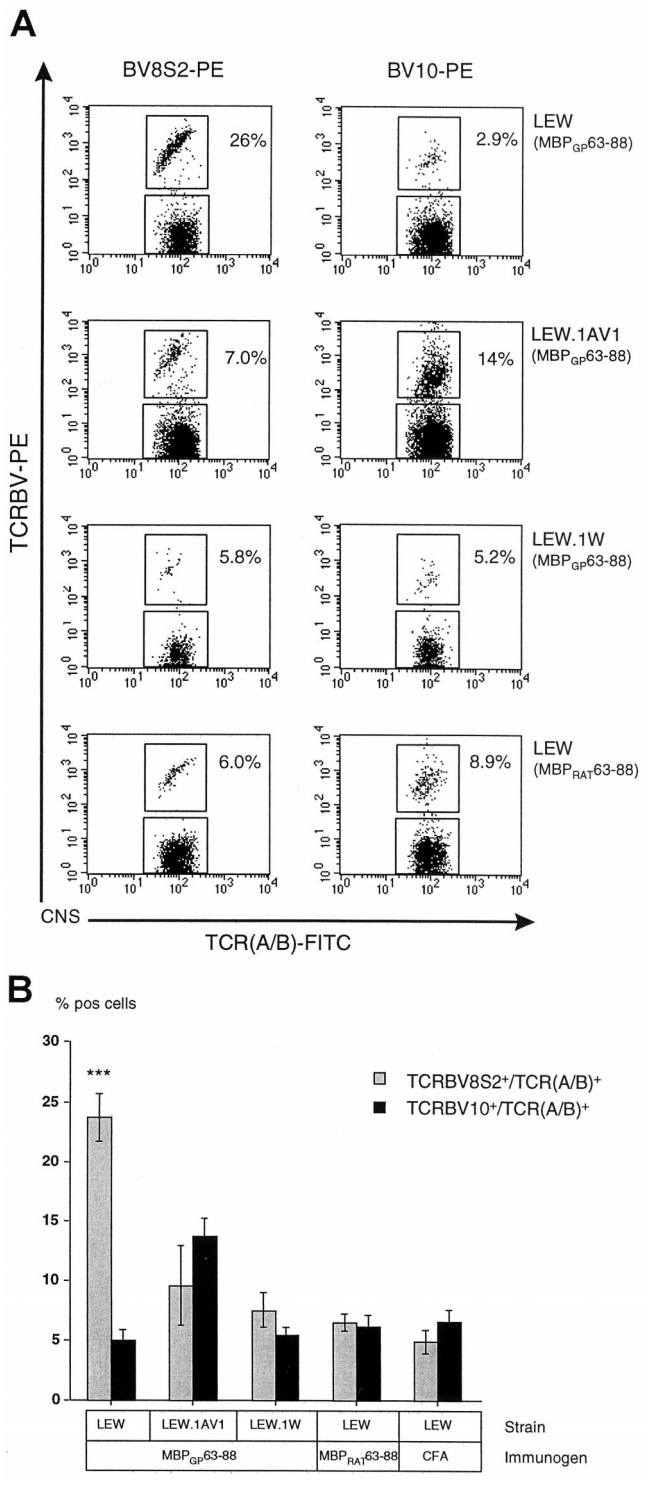

FIGURE 4. Frequencies of CNS-infiltrating TCRBV8S2$^+$ T lymphocytes in MBP63-88-immunized RT1 congenic LEW rat strains. *A* illustrates typical two-color flow cytometric analyses of TCRBV8S2$^+$/TCR(A/B)$^+$ and TCRBV10$^+$/TCR(A/B)$^+$ T cells recovered from the CNS of a randomly chosen individual from the groups of RT1 congenic LEW rats immunized with MBP$_{GP}$63-88 and from LEW rats immunized with MBP$_{RAT}$63-88, mentioned in *B*. *B* indicates the frequencies of TCRBV8S2$^+$/TCR(A/B)$^+$ and TCRBV10$^+$/TCR(A/B)$^+$ T cells recovered from the CNS of LEW ($n = 7$), LEW.1AV1 ($n = 5$), and LEW.1W ($n = 4$) rats immunized with MBP$_{GP}$63-88, and LEW rats ($n = 5$) immunized with MBP$_{RAT}$63-88 and CFA-injected LEW controls ($n = 4$), as determined by two-color FACS analyses. CNS-derived cells were recovered on day 12 p.i. Collection of CNS-infiltrating T lymphocytes, staining-procedures, and evaluation of data were performed as described in *Materials and Methods*. Each bar represents the mean ± SEM. *** indicates statistical significance ($p < 0.0003$).

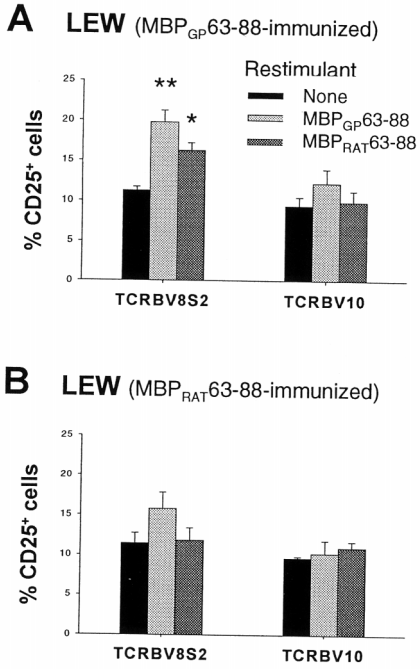

FIGURE 5. Co-expression of CD25 on TCRBV8S2$^+$ and TCRBV10$^+$ T cells from MBP$_{GP}$63-88-primed and MBP$_{RAT}$63-88-primed LEW rats after in vitro restimulation with MBP63-88 peptides. LN cells were obtained on day 12 p.i. and cultured for 48 h with or without MBP$_{GP}$63-88 or MBP$_{RAT}$63-88 (10 μg/ml). *A* depicts the frequencies of in vitro restimulated TCRBV8S2$^+$/CD25$^+$ and TCRBV10$^+$/CD25$^+$ T cells from draining LN of MBP$_{GP}$63-88-primed LEW ($n = 4$) and *B* from MBP$_{RAT}$63-88-primed LEW rats ($n = 4$) as determined by two-color FACS analyses. Collection of cells from draining LN, culture conditions, staining procedures, and evaluation of data were performed as described in *Materials and Methods*. Each bar represents the mean ± SEM. ** and * indicate statistical significance ($p < 0.01$ and $p < 0.05$).

levels after culture with MBP$_{GP}$63-88 and MBP$_{RAT}$63-88 (Fig. 6*B*). Even more conspicuous and discriminating MBP peptide responses were recorded by using production of the proinflammatory cytokine IFN-γ as the outread. Lymphoid cells from MBP$_{GP}$63-88-immunized LEW rats showed high numbers of IFN-γ-secreting cells after restimulation in vitro with MBP$_{GP}$63-88. Such cells were detected, although at lower numbers, also after culture with MBP$_{RAT}$63-88 (Fig. 6*C*). MBP$_{RAT}$63-88-immunized LEW rats had high numbers of IFN-γ-secreting cells already at low peptide concentrations after culture with MBP$_{GP}$63-88, while MBP$_{RAT}$63-88 stimulated stronger at higher peptide concentrations (Fig. 6*D*).

In vivo-depletion of encephalitogenic TCRBV8S2$^+$ T lymphocytes

To assess the clinical importance of TCRBV8S2$^+$ T cells, MBP$_{GP}$63-88-primed LEW and LEW.1AV1 rats and, in addition, MBP$_{RAT}$63-88-primed LEW rats were injected i.p. with anti-TCRBV8S2 mAb on day 8 p.i. Depletion was determined by FACS analysis of T cells from blood on day 9 p.i. In all cases, the reductions in numbers of TCRBV8S2$^+$ T cells exceeded 50% and the TCR densities on the remaining TCRBV8S2$^+$ T cells were markedly down-regulated (data not shown). As expected, the

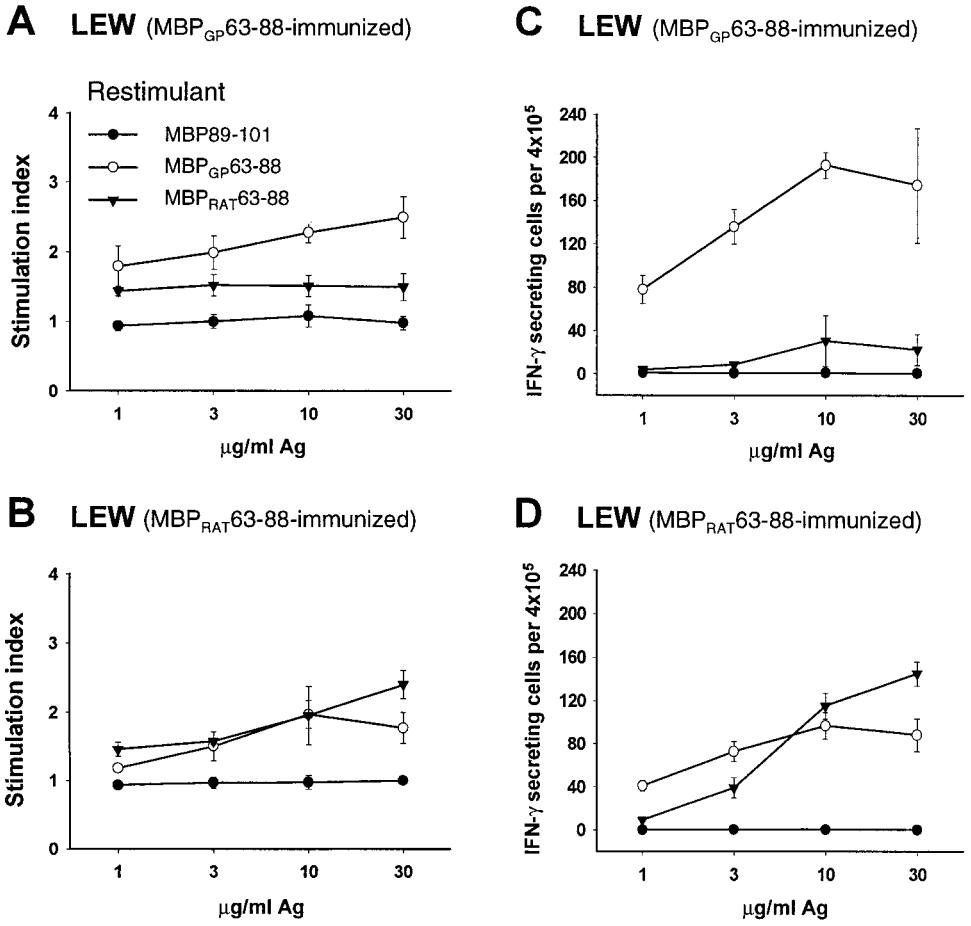

FIGURE 6. Ag-specific proliferation and enumeration of IFN-γ-secreting T cells from MBP$_{GP}$63-88-primed and MBP$_{RAT}$63-88-primed LEW rats. *A* depicts proliferative responses of T cells from draining LN of MBP$_{GP}$63-88-primed LEW rats ($n = 4$) and *B* from MBP$_{RAT}$63-88-primed LEW rats ($n = 4$). LN were obtained on day 12 p.i. and MNC were cultured for 72 h in triplicates in the presence of either MBP$_{GP}$63-88, MBP$_{RAT}$63-88, or MBP89-101 at various Ag concentrations or medium alone. T cell proliferation was assessed by [^{3}H]TdR incorporation during the last 12 h of culture. *C* shows numbers of IFN-γ-secreting cells per 4×10^5 MNC from spleens of MBP$_{GP}$63-88-primed LEW ($n = 4$) and *D* from MBP$_{RAT}$63-88-primed LEW rats ($n = 4$). Spleens were obtained on day 12 p.i. and MNC were cultured for 48 h in triplicates in the presence of either MBP$_{GP}$63-88, MBP$_{RAT}$63-88, or MBP89-101 at various Ag concentrations or medium alone. The ELISPOT assay was performed as described in *Materials and Methods*. Each line represents the mean stimulation index/number of IFN-γ-secreting cells ± SEM.

TCRBV10$^+$ T cell subset was unaffected (data not shown). Following injections with the anti-TCRBV8S2 mAb, only LEW rats pre-immunized with MBP$_{GP}$63-88 exhibited mitigation of the EAE disease course, as distinguished from both LEW rats pre-immunized with MBP$_{RAT}$63-88 ($p < 0.05$) and LEW.1AV1 rats pre-immunized with MBP$_{GP}$63-88 ($p < 0.05$) (Fig. 7).

Binding affinities of MBP$_{GP}$63-88 and MBP$_{RAT}$63-88 to purified RT1.B^l molecules

The RT1.B^l molecule is the restriction element for most encephalitogenic T cells in MBP63-88-induced EAE in LEW rats (29). To test whether the affinities of MBP$_{GP}$63-88 vs MBP$_{RAT}$63-88 for the groove of the RT1.B^l molecule may differ, which would at least partly offer an explanation for the variations in recruitment patterns of TCRBV8S2-expressing T cells, we performed competitive binding studies including the two MBP63-88 peptides and OX-6 affinity-purified RT1.B^l molecules (Fig. 8). RT1.B^l molecules were incubated with a biotinylated reference peptide (MBP$_{GP}$72-85; 500 nM) and serial dilutions of the respective relevant peptide (5 nM-250 μM). Practically equally strong inhibition of binding was observed for both MBP63-88 peptides (IC_{50}: MBP$_{GP}$63-88, 2.5 μM; MBP$_{RAT}$63-88, 0.7 μM). In comparison, the MBP$_{RAT/GP}$89-101 peptide, which has been reported to associate with the RT1.D^l

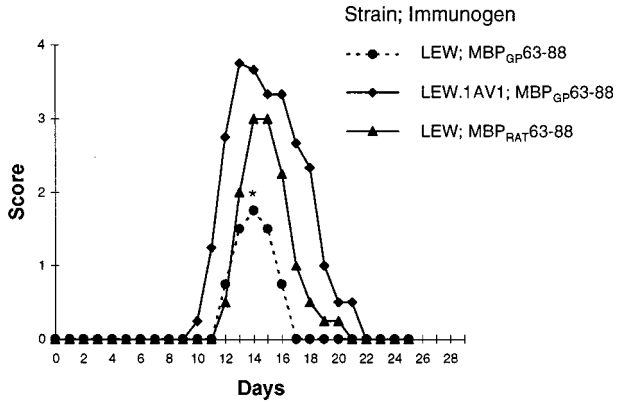

FIGURE 7. In vivo depletion of TCRBV8S2$^+$ T cells. All RT1 congenic LEW rat strains were initially immunized with 200 μg of the relevant MBP63-88 peptides, followed by injection with 150 μg of the TCRBV8S2-specific mAb, R78, on day 8 p.i. EAE disease courses of R78-injected LEW ($n = 4$) and LEW.1AV1 ($n = 4$) rats pre-immunized with MBP$_{GP}$63-88, and of R78-injected LEW rats ($n = 4$) pre-immunized with MBP$_{RAT}$63-88 are depicted. The efficacy of R78 treatment was determined on day 9 p.i., as described in *Results*. * indicates statistical significance ($p < 0.05$).

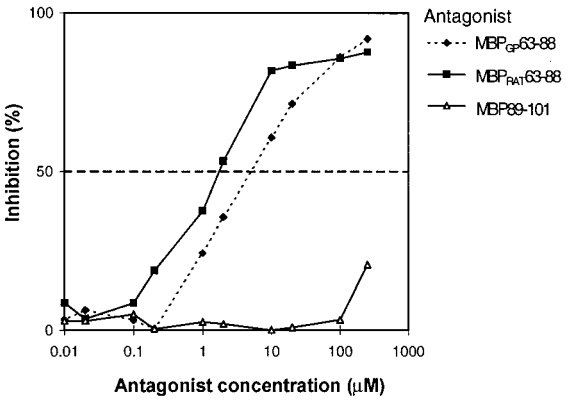

FIGURE 8. Binding affinities of MBP$_{GP}$63-88 and MBP$_{RAT}$63-88 to purified RT1.B^{l} molecules. OX-6 affinity-purified RT1.B^{l} molecules were incubated with the biotinylated MBP$_{GP}$72-85 peptide (500 nM) and increasing concentrations (5 nM-250 μM) of the respective relevant peptide (antagonist), MBP$_{GP}$63-88 or MBP$_{RAT}$63-88. IC$_{50}$ values for MBP$_{GP}$63-88 and MBP$_{RAT}$63-88 were 2.5 μM and 0.7 μM, respectively. For comparison, the RT1.D^{l}-restricted MBP$_{GP/RAT}$89-101 peptide was included as a negative control antagonist.

allelic product (30), showed weak competitive capacity and only at high molar concentrations (IC$_{50}$>250 μM).

Discussion

Differences in LEW rat EAE T cell responses to self- and nonself-MBP have been thoroughly investigated with regard to parameters such as specificity, immunogenicity, antigenicity, and cross-reactivity (31, 32). To date, however, potential variations in relative distribution patterns of the TCR repertoires expressed by MBP-activated T cell subsets have not been elucidated. Our data revealed that the TCRBV repertoire induced by the autologous MBP$_{RAT}$63-88 peptide is distinct from that induced by its heterologous counterpart, MBP$_{GP}$63-88, although both peptides generated similar EAE disease severity. Hence, we established that the preferential TCRBV8S2 usage by encephalitogenic T cells of the LEW rat is based on the specific combination of endogenous expression of the RT1.B^{l} allelic product and exogenous initiation of the heterologous MBP$_{GP}$63-88 peptide.

Our flow cytometric approach confers improved precision and sensitivity on quantitative analyses of activation and expansion levels of T cell subsets expressing particular TCRBVs. It also has the advantage that the results would not be biased by strong selective pressures that may affect data obtained from long-term growth of MBP-specific T cell lines and clones, hitherto used for characterizations of TCR repertoires (11). In draining LN and blood from LEW rats immunized with MBP$_{GP}$63-88, we detected increased numbers of TCRBV8S2^{+}/CD25^{+} T cells. Additionally, in the blood, but not in the LN, significant expansions of TCRBV8S2^{+} T cells were detected and, interestingly, roughly 30% of the CNS-derived T lymphocytes expressed the TCRBV8S2 product. These observations indicate that Ag-specific activation of TCRBV8S2^{+} T cells in the draining LN leads to a ready release of these lymphocytes into the bloodstream. On passing through CNS vessels, and because they are in an activated state expressing appropriate sets of adhesion molecules, the TCRBV8S2^{+} T cells fulfill the requirements for being able to cross the blood-brain barrier and thus accumulate at the site at which MBP is exposed (1). In contrast to meningeal and cerebrospinal fluid-derived T cells, a considerable quantity of T cells in parenchymal CNS lesions appears to undergo Ag-specific apoptosis (33, 34), which may explain why only a minority of infiltrating cells recovered from the CNS parenchyma is Ag-specific (35). The fact that depletion of TCRBV8S2^{+} T cells led to amelioration of EAE (Ref. 36, and the present study) and that our CNS data are based on cells recovered from surface areas of the CNS and the meninges, indicate that a substantial fraction of CNS-associated T cells might be encephalitogenic and Ag specific.

The MBP$_{GP}$63-88 and MBP$_{RAT}$63-88 peptides differ in three amino acid positions. Positions 63 and 64 contain alanine residues in the MBP$_{GP}$63-88 sequence, whereas MBP$_{RAT}$63-88 has histidine at position 63 and threonine at position 64. Both positions are located outside the minimal core region of the sequence important for induction of EAE (29). Inside the core region, at position 79, threonine (T$_{79}$) in MBP$_{RAT}$63-88 is replaced by serine (S$_{79}$) in MBP$_{GP}$63-88. Consequently, this single amino acid replacement most likely constitutes the basis of the differential MBP63-88-specific TCRBV repertoires. In support of this inference, it has previously been reported that a variant of the moth cytochrome *c*-derived 88-103 peptide, in which glutamate is substituted for threonine in position 102, converted a T cell response characterized by TCRBV3 overusage into a T cell response encompassing principally normally distributed TCRBV products (18).

Potentially, a reciprocal amino acid exchange in a given peptide sequence can affect the distribution of the epitope-specific TCR repertoire by contributing to differences either in binding affinities of the two peptides to the restricting MHC class II molecule or in selective involvement in recognition of the respective relevant amino acid, exposed as a TCR contact residue. The ligand-binding motif of the RT1.B^{l} molecule has recently been defined by combining the pool sequencing approach with sequencing of individual ligands (37). Interestingly, the side chains of the two alternative core amino acids of the MBP63-88 sequence, T$_{79}$ and S$_{79}$, both appear to qualify as anchor residues for pocket 6 of the allele-specific RT1.B^{l} groove. However, another recently published study, in which a panel of alanine-substituted MBP$_{GP}$72-85 analogues was tested for each member's relative binding strength to the RT1.B^{l} motif, indicated that T$_{79}$ and S$_{79}$ would rather act solely as TCR contact residues (38). Nevertheless, despite the fact that the single T$_{79}$/S$_{79}$ substitution has to be considered conservative and that competitive binding studies of MBP$_{GP}$63-88 and MBP$_{RAT}$63-88 excluded differences in their affinities for the RT1.B^{l} molecule, the two alternative peptides have distinct capacities for evoking differential TCR repertoire patterns. It can thus be deduced that an utmost minimum structural difference in the MBP63-88 core sequence may lead to radical conformational changes in the TCR-identifiable part of the peptide/RT1.B^{l} complex. On the assumption that T$_{79}$ and S$_{79}$ interact with pocket 6 of the RT1.B^{l} groove, their differential contribution to the three-dimensional structure of the peptide/RT1.B^{l} complexes can be accounted for by distinct specific effects on the orientation of the identical flanking core TCR contact amino acids of the MBP63-88 peptide. Alternatively, though less likely, T$_{79}$ and/or S$_{79}$ may not only be involved in an anchoring function but also be partly exposed outwards, allowing them to act as direct TCR contact units. However, ultimate elucidation of the exact role of each amino acid core residue of MBP$_{GP}$63-88 and MBP$_{RAT}$63-88, respectively, in the TCR/peptide/RT1.B trimolecular complex awaits its resolution, possibly by virtue of employing x-ray crystallography.

The peripheral TCR repertoire is established by the effects of phenomena such as intra- and extrathymic selection events (39), multiple allelism of TCR-coding gene segments (40), and endogenous expression of superantigens (41). In addition, incessant challenges by exogenous immunogens in the periphery impose continuous modifications on the composition of the pre-immune TCR

repertoire (42). By the use of inbred MHC congenic LEW rats, kept under identical physical conditions, we can preclude germline-encoded TCR polymorphism, occurrence of endogenous superantigens, and unintentional environmental influences from acting as factors contributing to the over-representation of the TCRBV8S2$^+$ T cell subset in the MBP$_{GP}$63-88-primed LEW rat.

The capacity for eliciting preferential peripheral activation of MBP$_{GP}$63-88-specific TCRBV8S2$^+$ T cells may depend on central tolerance induction. Since only LEW rats with intact thymi exhibit preferential TCRBV8S2 usage (39), it can be presumed that the autoreactive MBP$_{RAT}$63-88-specific TCR repertoire is shaped to a high degree by self-peptides presented by RT1.B^l molecules on thymus-associated APCs during the course of T cell maturation (43). The tolerogenic peptides should originate from the self-MBP itself (44) and/or other self-molecules, such as RT1^l Ags, sharing homology sequences with self-MBP (45). According to the affinity/avidity selection model of thymocyte development, it can be argued that a substantial portion of the TCRBV8S2$^+$ pre-T cells escape negative selection by binding at low avidity to the self-peptide/RT1.B^l complex (46, 47). These pre-T cells will eventually reach a full immunocompetent state, thus constituting part of the naive peripheral T cell pool, which can become specifically activated through high-avidity binding to the nonself-MBP$_{GP}$63-88/self-RT1.B^l complex. After immunogen challenge, the immune system operates by favoring T cells with high avidity for Ag (48). As a consequence, preferential recruitment of MBP$_{GP}$63-88-specific T cells expressing TCRBV8S2 may prevail.

Differences in disconcordance rates for MS within monozygotic (70%) and dizygotic (95%) twins, respectively, argue for involvement of both genetic predispositions and environmental factors in contributing to the probability of developing MS (49). Various infectious agents have been hypothesized to be a crucial etiologic factor. A plausible mechanism of induction of MS may thus be based on a multistep molecular mimicry hypothesis, in which microorganisms trigger autoimmune reactions by providing immunogenic nonself-peptides, cross-reactive with self (50, 51). Since MBP$_{GP}$63-88 can be considered the equivalent of any foreign peptide sharing homology sequences with a self-molecule, the LEW rat EAE may be a valid model for studying the T cell-related immunopathogenicity of MS. Admittedly, the situation in humans may be more complex in view of its chronicity and B cell-mediated pathogenesis (52). Our LEW EAE data suggest that thymic selection over the RT1^l-encoded allele-specific molecules in conjunction with the collection of available self-peptides shapes a peripheral pre-immune TCR repertoire encompassing low-avidity anti-self-TCRBV8S2$^+$ T cells. Following systemic challenge, these T cells will become prevalently activated and expanded through selective high-avidity recognition of the MBP$_{GP}$63-88/RT1^l class II complex. This series of events may result in autoaggressive cross-reactivity with the MBP$_{RAT}$63-88/RT1^l class II complex, thus being instrumental for induction of EAE. Indeed, we observed that MBP$_{GP}$63-88-primed TCRBV8S2$^+$ T cells responded to MBP$_{RAT}$63-88 in vitro, and there were immune cross-reactivities both with proliferation and proinflammatory cytokine production. A similar multistep scenario may underlie the skewed TCRBV repertoire described in certain HLA-Dw2$^+$ MS patients (7–9). By cross-reactivity with immunogenic peptides derived from common bacteria and viruses, low-avidity, TCR repertoire-restricted self-MBP-reactive T cells may become activated and expanded (50, 51). Thus, as in the LEW rat EAE model, nonself, self-mimicking exogenous Ag-derived sequences would participate in peptide/MHC complexes recognized at high avidity by autoreactive encephalitogenic T cells expressing particular TCRBVs.

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Publikation 2

Protective DNA vaccination against organ-specific autoimmunity is highly specific and discriminates between single amino acid substitutions in the peptide autoantigen

Robert Weissert*†‡§, Anna Lobell‡¶, Katrien L. de Graaf*†, Sana Yousif Eltayeb*, Roland Andersson¶, Tomas Olsson*, and Hans Wigzell¶

*Neuroimmunology Unit, CMM L8:04, Karolinska Hospital, S-17176 Stockholm, Sweden; ¶Microbiology and Tumorbiology Center, Karolinska Institute, S-17177 Stockholm, Sweden; and †Department of Neurology, University of Tuebingen, Hoppe-Seyler-Strasse 3, D-72076 Tuebingen, Germany

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DNA vaccines that encode encephalitogenic sequences in tandem can protect from subsequent experimental autoimmune encephalomyelitis induced with the corresponding peptide. The mechanism for this protection and, in particular, if it is specific for the amino acid sequence encoding the vaccine are not known. We show here that a single amino acid exchange in position 79 from serine (nonself) to threonine (self) in myelin basic protein peptide MBP68–85, which is a major encephalitogenic determinant for Lewis rats, dramatically alters the protection. Moreover, vaccines encoding the encephalitogenic sequence MBP68–85 do not protect against the second encephalitogenic sequence MBP89–101 in Lewis rats and vice versa. Thus, protective immunity conferred by DNA vaccination exquisitely discriminates between peptide target autoantigens. No bystander suppression was observed. The exact underlying mechanisms remain elusive because no simple correlation between impact on *ex vivo* responses and protection against disease were noted.

DNA vaccination can protect from autoimmune diseases. The mechanisms involved are poorly understood and may well differ between different DNA constructs and ways of application. A mechanism involving immune deviation was suggested in experiments in which DNA vaccination with a construct encoding a T cell receptor (TCR) β chain resulted in a T helper 2 (Th2) shift of encephalitogenic T cells, thus protecting Pl/J mice from experimental autoimmune encephalomyelitis (EAE) (1). With another approach, a form of anergy was implied, because myelin basic protein (MBP) peptides in tandem as DNA vaccine in contrast resulted in reduction of a Th1 immune response of encephalitogenic T cells without a measurable increase of Th2 type responses (2).

Immune responses after DNA vaccination have been analyzed more thoroughly when applied for infectious diseases (3). Sequences encoding foreign antigenic stretches induce strong, antigen-specific T and B cell responses that are able to protect from subsequent disease. MHC class I-restricted $CD8^+$ T cell responses mainly have been described, whereas less is known about MHC class II-restricted $CD4^+$ T cell responses. Even in these more well studied situations, there is little information on the specificity requirements of the vaccines. Does protective vaccination also act on closely related peptides both in context of infectious disease and autoimmunity? Crossreactivity of the resulting immune response would be plausible in view of the recently emphasized high degeneracy of T cell recognition of antigens (4, 5). These questions are of importance for several reasons. First, DNA vaccination with foreign antigens might lead to unwanted recognition of self-antigens with autoimmunity as a consequence. Second, DNA vaccination against autoimmunity might affect natural immunity against infections. Third, in human autoimmune disease, the disease-promoting triggers are not defined with regard to exact amino acid sequence (exogenous host-mimicking vs. purely self) (6). Furthermore, epitope spreading might result in a disease-promoting immune response (7). Thus, if DNA vaccines are to be used therapeutically or prophylactically, one would need to know the potential degeneracy of any protective immune response and whether any bystander suppression, perhaps through immune deviation, might affect both closely related and strikingly dissimilar peptide responses. With this background, we study here specificity requirements for DNA vaccines in the well defined MBP peptide-induced Lewis rat EAE model.

The autologous MBP peptide $MBP_{RAT}68–85$ and the heterologous MBP peptide $MBP_{GP}68–85$ differ in a single amino acid exchange from threonine (T) to serine (S) in position 79. We had shown recently that this minor difference has a strong impact on the expansion on $TCRBV8S2^+$ T cells (8). Furthermore, we had shown that $MBP_{GP}68–85$- induced EAE can be protected from by immunization with a DNA vaccine consisting of tandem repeats of $MBP_{GP}68–85$ and targeting of the gene product to IgG (2). Now, we wanted to investigate whether DNA encoding the autologous vs. the heterologous MBP peptides could protect against subsequent challenge with both peptides and how different encephalitogenic stretches of MBP can protect against each other. Furthermore, we investigated whether DNA encoding the whole rat MBP 21.5-kDa sequence can protect against peptide-induced EAE.

Materials and Methods

Rats. Lewis (LEW) rats were obtained from Harlan Netherlands (Zeist, The Netherlands). All rats were housed under specific pathogen-free conditions to keep the influence of additional environmental factors as well as immunization as low as possible. They were checked routinely for specific pathogens. In all experiments, female rats, 8–9 weeks of age, were used.

IMMUNOLOGY

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin-oligodendrocyte glycoprotein; TCR, T cell receptor; Th, T helper; MNC, mononuclear cell(s); LEW, Lewis.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database [accession no. AJ132898 (cDNA encoding 21.5-kDa isoform of rat MBP)].

‡R.W. and A.L. contributed equally to this work.

§To whom reprint requests should be addressed at the † address. E-mail: robert.weissert@uni-tuebingen.de.

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Table 1. Peptide sequences

$MBP_{GP}68–85$	HYGSLPQKSQR**S**QDENPV
$MBP_{GP}63–88$	AARTTHYGSLPQKSQR**S**QDENPVVHF
$MBP_{RAT}68–85$	HYGSLPQKSQR**T**QDENPV
$MBP_{RAT}63–88$	HTRTTHYGSLPQKSQR**T**QDENPVVHF
MBP89–101	VHFFKNIVTPRTP
MOG37–54	VGWYRSPFSRVVHLYRNG

Induction and Evaluation of EAE. The rats were anaesthetized by inhalation anesthesia with methoxyflurane (Metofane; Pitman–Moore, Mundelein, IL) and injected intradermally at the base of the tail with a total volume of 200 μl of inoculum, consisting of 100 μg of peptide emulsified (1:1) with complete Freund's adjuvant (Sigma) containing 500 μg of heat-inactivated mycobacterium tuberculosis (strain H 37 RA from Difco).

Animals were scored for clinical signs of EAE and weighed daily up to 25 days postimmunization. The signs were scored as follows: grade 1, tail weakness or tail paralysis; grade 2, hind leg paraparesis or hemiparesis; grade 3, hind leg paralysis or hemiparalysis; grade 4, complete paralysis (tetraplegy), moribund state, or death.

Synthetic Peptides and Antigens. The synthetic peptides were synthesized by Fmoc/HBTU strategy (Å. Engström, Department of Medical and Physiological Chemistry, University of Uppsala, Sweden). Peptides were purified by reversed-phase chromatography and, subsequently, analyzed by plasma desorption MS. The degree of purity of the used peptides was >99%. The peptide sequences are shown in Table 1. $_{GP}$MBP, purified MBP from central nervous system tissue of guinea pigs, was bought from Sigma.

Fractionation and Cultivation of Mononuclear Cells from Spleen. Spleens were dissected out under deep anesthesia and disrupted, and mononuclear cells (MNC) were washed twice in DMEM (Life Technologies, Paisley, Scotland), resuspended in complete medium (CM) containing DMEM supplemented with 1% rat serum, 1% penicillin/streptomycin (Life Technologies), 1% glutamine (Life Technologies), and 50 μM 2-mercaptoethanol (Life Technologies), and flushed through a 70-μm plastic strainer (Falcon; Becton Dickinson). Red blood cells were lysed with lysing buffer, consisting of 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA adjusted to pH 7.4.

MNC were cultured at a concentration of 2×10^6 cells/ml in either 96-well, round-bottomed microtiter plates (Nunc) with 100 μl of cell suspension per well or in flat-bottomed nitrocellulose plates for enzyme-linked immunospot assay (ELISPOT) (MAHA; Millipore) with 200 μl of cell suspension per well at 37°C in a humidified atmosphere containing 5% CO_2.

Assays of Antigen-Induced Proliferation. All proliferative experiments were performed in triplicate in 96-well, round-bottomed microtiter plates. MNC (2×10^5/well) in 100 μl of CM were cultured with or without the relevant Ag for 60 h and, subsequently, pulsed with 0.5 mCi of [^{3}H]thymidine (Amersham Pharmacia) per well for 12 h. DNA was collected on glass-fiber filters (Skatron, Sterling, VA), and [^{3}H]thymidine-incorporation was measured in a β-counter (Beckman Coulter).

Enumeration of Cells Secreting Antigen-Specific IFN-γ. To enumerate T cells secreting IFN-γ after Ag exposure, an ELISPOT method was used (8). Nitrocellulose-bottomed, 96-well plates were coated with the mouse mAb DB1 (a generous gift of Peter van der Meide, TNO Primate Centre, Rijswijk, The Netherlands), which reacts with rat IFN-γ. After washing with PBS, the plates were blocked with DMEM containing 5% FCS (Life Technologies). Cells (4×10^5/well) in 200 μl of CM were added to the plates and incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. For each Ag, triplicate determinations were performed. Cells then were discarded and plates were washed four times with PBS. Secreted and bound IFN-γ was visualized with biotinylated DB12 (also a generous gift of Peter van der Meide), which has a binding site on IFN-γ other than DB1—avidin-biotin peroxidase (Vector Laboratories)—and, subsequently, by staining with carbazole (Sigma).

Cytokine ELISA. ELISA kits for detection of secreted IL-4 and IL-10 were purchased from BioSource International (Camarillo, CA). Supernatants from MNC from spleens, which had been incubated at a concentration of 2×10^6 cells/ml with or without relevant antigens or Con A, were analyzed (2). The procedure was performed as recommended by the manufacturer.

Plasmid Construction. For pZZ/MBP_{GP}68–85, pZZ/MBP_{RAT}68–85, and pZZ/MBP89–101, a 94-bp fragment containing a murine heavy chain IgG signal sequence (ss) was ligated upstream and in-frame of a 385-bp fragment encoding ZZ (2). Directly downstream of the coding sequence ZZ and upstream of the stop codon, seven *Ava*I-*Ava*I fragments encoding MBP_{GP}68–85 or MBP89–101 or six *Ava*I-*Ava*I fragments encoding MBP_{RAT}68–85 were ligated in-frame. cDNA encoding a 21.5-kDa isoform of rat MBP (GenBank accession no. AJ132898), which is formed by alternative splicing of MBP mRNA, was cloned by standard reverse transcription–PCR and PCR procedure. After sequencing, obtaining two identical sequences from different PCRs, a single copy of the MBP_{RAT}21.5-coding cDNA was cloned into expression vector pCI, downstream of ZZ. For pZZ, a fragment containing ss and ZZ in frame was cloned into pCI (Promega). Expression is driven by an immediate/early human CMV enhancer/promoter. The *Escherichia coli* host was XL1-Blue (Stratagene).

Plasmid Preparation. Plasmid DNA was prepared by Qiagen plasmid preparation protocol. Endotoxins were removed in an additional step (Endofree buffer set; Qiagen) (2).

Plasmid DNA Injections and Cardiotoxin Pretreatment. Five- to 6-week-old LEW ($RT1^l$) male rats were injected with 100 μl of 10 μM cardiotoxin (Latoxan, Rosans, France) into the musculi (Mm.) tibialii and Mm. gastrocnemii. Seven days later, the rats were injected with 800 μg of DNA vaccine at 2.0 μg/ml in PBS, divided into four 200-μg injections administered in the Mm. tibialii and Mm. gastrocnemii (2).

Statistics. Student's t test was used for normally distributed variables. When the data did not fulfill the criteria of being normally distributed, nonparametric statistics (Mann–Whitney U test) were used.

Results

Exquisite Epitope Specificity of Protection by DNA Vaccination. As shown in Table 2 in experiment 1, only pZZ/MBP_{GP}68–85 protected from MBP_{GP}68–85-induced EAE ($P < 0.01$), whereas DNA constructs encoding pZZ/MBP_{RAT}68–85 and pZZ/MBP_{RAT}21.5 failed to result in significant protection. Vice versa, pZZ/MBP_{RAT}68–85 resulted in significant protection against challenge with MBP_{RAT}68–85 ($P < 0.01$), whereas constructs pZZ/MBP_{GP}68–85 and pZZ/MBP_{RAT}21.5 did not. These results demonstrate a high level of antigen specificity in protection by DNA vaccination.

In experiment 2 we investigated how far pZZ/MBP_{GP}68–85 can protect against induction of disease with the second dominant epitope in the LEW rat, MBP89–101. As shown, there was no protective effect, but there was significant protection against

Table 2. Mean accumulated EAE score

	Treatment	Immunogen							
		$MBP_{GP}68–85$	*P*	$MBP_{RAT}68–85$	*P*	MBP89–101	*P*	$_{GP}MBP$	*P*
Experiment 1	$pZZ/MBP_{GP}68–85$	3.5	<0.01	8.0	NS				
	$pZZ/MBP_{RAT}68–85$	8.1	NS	3.6	<0.01				
	$pZZ/MBP_{RAT}21.5$	8.8	NS	8.3	NS				
	pZZ	12.9		11.8					
Experiment 2	$pZZ/MBP_{GP}68–85$	7.0	<0.001			8.5	NS	7.6	<0.01
	pZZ/MBP89–101	11.8	NS			5.9	<0.05	14.8	NS
	$pZZ/MBP_{GP}68–85$ and pZZ/MBP89–101	8.5	<0.05			7.2	NS	9.1	<0.05
	pZZ	13.9				10.1		13.9	

The table shows mean accumulated EAE score in relation to DNA vaccine and subsequent immunization with either MBP peptide $MBP_{GP}68–85$, $MBP_{RAT}68–85$, MBP89–101, or full length $_{GP}MBP$. There was a high specificity of the protective effect of the DNA vaccine. Each group represents eight rats. Vaccinations, immunizations, and scoring were performed as described in *Materials and Methods. P* values relate to statistical comparisons with the indicated DNA vaccine in relation to pZZ.

EAE induction with $_{GP}MBP$ ($P < 0.05$). On the other hand, the construct pZZ/MBP89–101 protected against disease induced with the peptide MBP89–101 ($P < 0.05$), but not against disease induced with $MBP_{GP}68–85$. A mixture of both pZZ/$MBP_{GP}68–85$ and pZZ/MBP89–101 ameliorated EAE induced with $MBP_{GP}68–85$ ($P < 0.05$) and $_{GP}MBP$ ($P < 0.05$). In contrast, pZZ/MBP89–101 did not protect against $_{GP}MBP$.

Cellular Responses After Vaccination with Different MBP Constructs and Subsequent Challenge with MBP Peptides. Immune responses to MBP and myelin-oligodendrocyte glycoprotein (MOG) peptides after DNA vaccination with different MBP constructs and subsequent immunization with MBP peptides by proliferation and ELISPOT assay for detection of IFN-γ-secreting cells and cytokine ELISAs for IL-4 and IL-10 were monitored.

As shown, DNA vaccination with pZZ and subsequent challenge with $MBP_{GP}68–85$ resulted in a strong, proliferative response against $MBP_{GP}68–85$ (Fig. 1*A*) and a lower response to $MBP_{RAT}68–85$ (Fig. 1*C*), whereas disease induction with $MBP_{RAT}68–85$ resulted in comparable responses to both peptides (Fig. 1 *B* and *D*). This was also the case for the number of IFN-γ-secreting cells as assessed by ELISPOT with higher

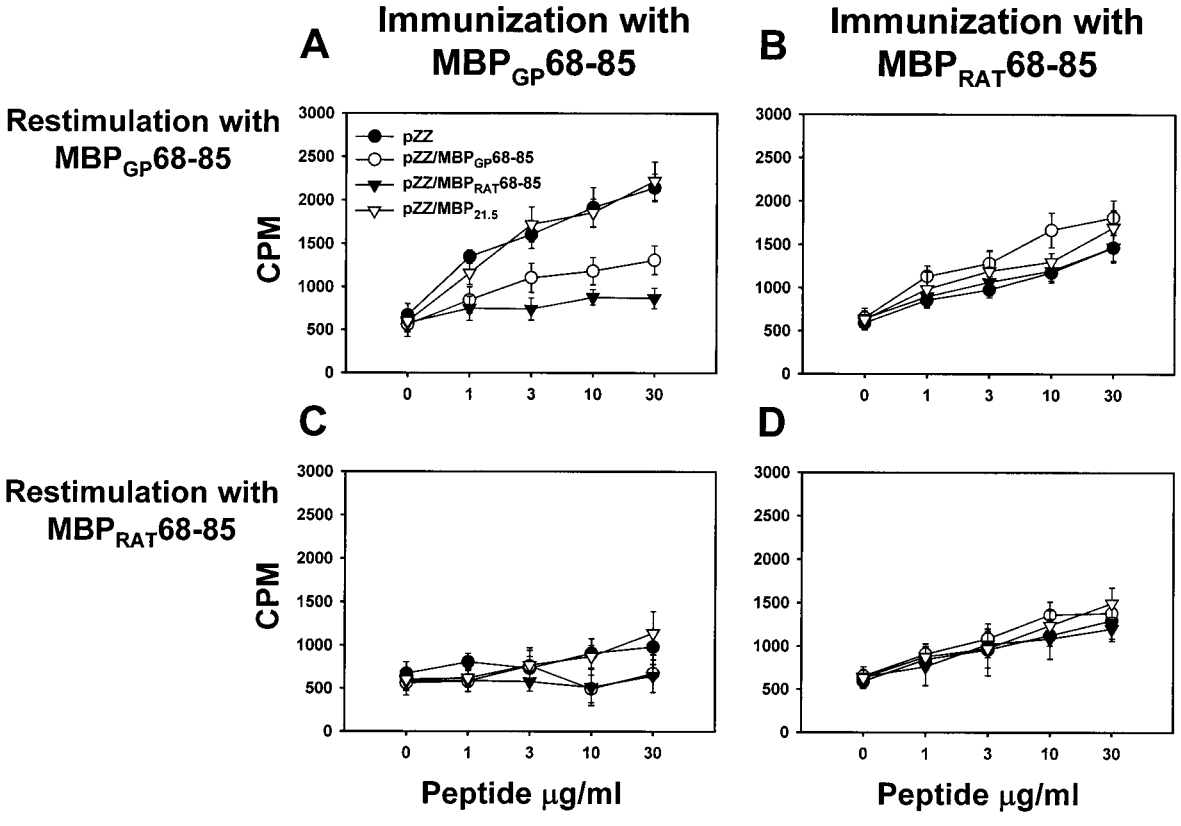

Fig. 1. Ag-specific proliferation of T cells from $MBP_{GP}68–85$ (*A* and *C*) and $MBP_{RAT}68–85$ (*B* and *D*) primed LEW rats that had received different DNA constructs 4 weeks before immunization i.m. as indicated in *Materials and Methods*. Each value represents mean values (±SEM) from four LEW rats. Splenocytes were obtained on day 12 postimmunization, and MNC were cultured for 72 h in triplicates in the presence of either $MBP_{GP}68–85$ (*A* and *B*) or $MBP_{RAT}68–85$ (*C* and *D*) at various antigen concentrations or medium alone. Stimulation with MBP89–101 or MOG37–54 did not result in cpm values over background (data not shown). T cell proliferation was assessed by [^{3}H]thymidine incorporation during the last 12 h of culture.

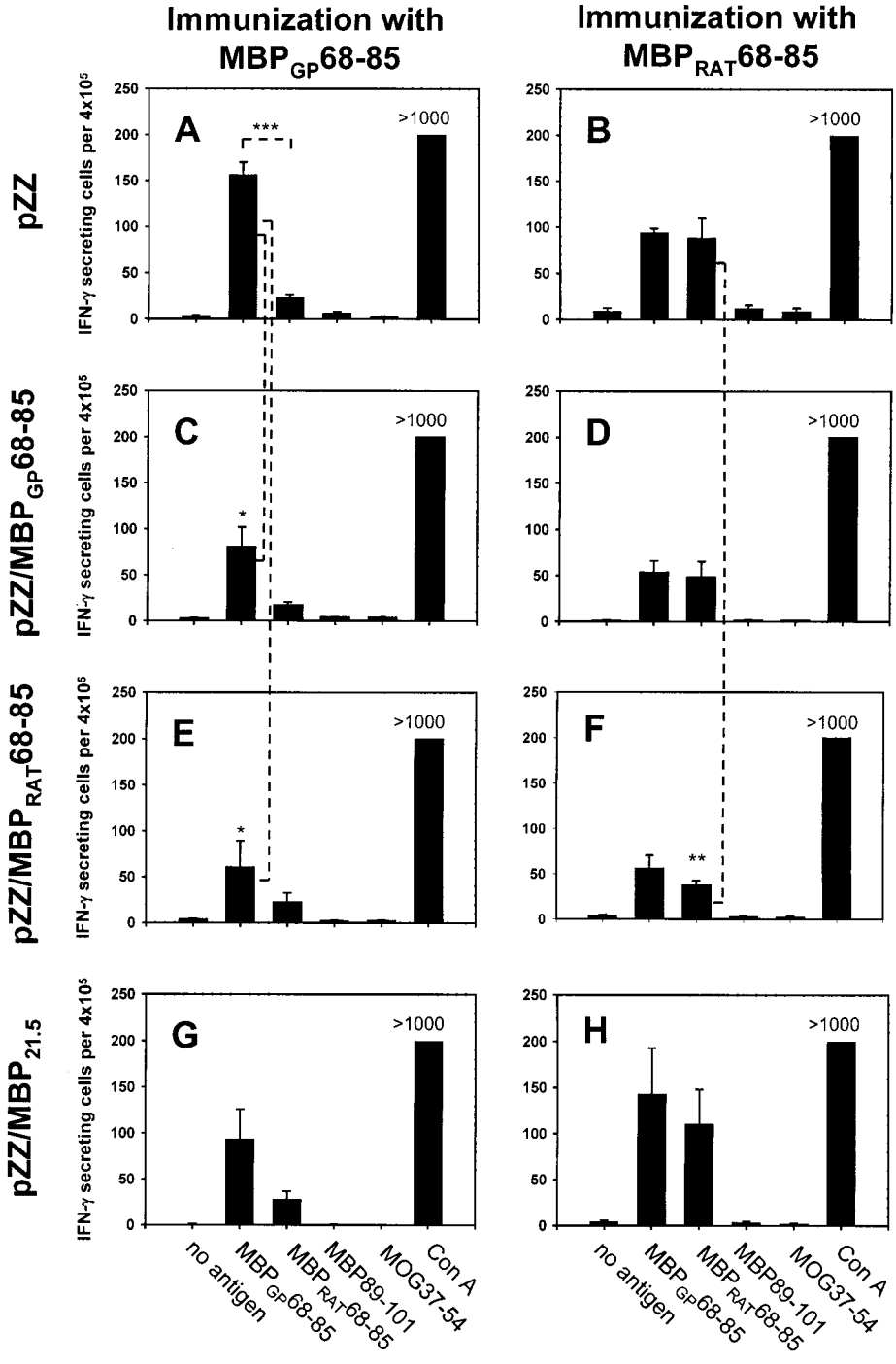

Fig. 2. Enumeration of cells secreting Ag-specific IFN-γ of T cells from MBP$_{GP}$68–85- and MBP$_{RAT}$68–85-primed LEW rats that had received different DNA constructs 4 weeks before immunization i.m. as indicated in *Materials and Methods*. Responses are shown after DNA vaccination with pZZ (*A* and *B*), after vaccination with pZZ/MBP$_{GP}$68–85 (*C* and *D*), after vaccination with pZZ/MBP$_{RAT}$68–85 (*E* and *F*), and after vaccination with pZZ/MBP$_{RAT}$21.5 (*G* and *H*). Each value represents data from four LEW rats. Spleens were obtained on day 12 postimmunization, and MNC were cultured for 48 h in triplicate in the presence of either MBP$_{GP}$68–85, MBP$_{GP}$63–88, MBP$_{RAT}$68–85, MBP$_{RAT}$63–88, MBP89–101, or MOG37–54 at a concentration 10 μg/ml peptide, medium alone, or Con A at a concentration of 3 μg/ml. Asterisks indicate significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

numbers of such cells after *in vitro* stimulation of MBP$_{GP}$68–85-immunized rat spleen cells with MBP$_{GP}$68–85 compared with MBP$_{RAT}$68–85 (Fig. 2*A*; $P < 0.001$). Equal numbers of such cells were noted after immunization with MBP$_{RAT}$68–85 and subsequent *in vitro* stimulation with either MBP$_{GP}$68–85 or MBP$_{RAT}$68–85 in pZZ-vaccinated rats (Fig. 2*B*).

Compared with DNA vaccination with the control construct pZZ, vaccination with pZZ/MBP$_{GP}$68–85 followed by immunization with MBP$_{GP}$68–85 led to a reduced proliferative capacity of MBP$_{GP}$68–85-reactive cells (Fig. 1*A*). In contrast, the proliferative capacity after immunization with MBP$_{RAT}$68–85 was not affected (Fig. 1*B*). Both constructs pZZ/MBP$_{GP}$68–85

and pZZ/MBP_{RAT}68–85 led, after immunization with MBP_{GP}68–85, to a reduction of IFN-γ-secreting cells induced by MBP_{GP}68–85 (both $P < 0.05$). pZZ/MBP_{RAT}21.5 encoding the full-length rat MBP 21.5-kDa molecule did not result in a decrease of MBP_{GP}68–85-reactive cells.

DNA vaccination with pZZ/MBP_{GP}68–85 and pZZ/MBP_{RAT}68–85 compared with pZZ, all subsequently immunized with MBP_{RAT}68–85, did not result in significant reduction of IFN-γ-secreting cells reactive for MBP_{GP}68–85 (Fig. 2 *B*, *D*, and *F*). pZZ/MBP_{GP}68–85 also did not lead to reduction of MBP_{RAT}68–85-reactive cells compared with pZZ (Fig. 2 *B* and *D*). In contrast, vaccination with pZZ/MBP_{RAT}68–85 resulted in significant reduction of MBP_{RAT}68–85-reactive cells secreting IFN-γ compared with vaccination with pZZ ($P < 0.01$) (Fig. 2 *B* and *F*). There were no differences in proliferative responses (Fig. 1*D*). We did not measure an effect of pZZ/MBP_{RAT}21.5 compared with pZZ (Figs. 1 and 2).

We never found any measurable *in vitro* response over background toward MBP89–101, the second minor dominant epitope in MBP for the LEW rat, or to MOG37–54, the dominant MOG epitope in the LEW rat (Fig. 2; not shown for proliferative data). There was no measurable antigen-induced IL-4 or IL-10 in ELISA after stimulation with peptides except for that for the mitogen Con A (data not shown).

Discussion

We demonstrate that (*i*) there is an exquisite epitope specificity of DNA vaccination, (*ii*) protection from disease is associated with reduction of T cell reactivity from spleen to the disease-inducing peptide, (*iii*) DNA vaccination with sequences for a certain myelin peptide does not cross-tolerize against other encephalitogenic MBP or MOG peptides, and (*iv*) DNA encoding the dominant encephalitogenic stretch in a high copy number has a higher protective potential compared with DNA encoding the full-length autoantigen in a single copy number.

We consider two principle mechanisms by which DNA vaccination with autoantigens could act protectively through deletion or active suppression of antigen-specific T cell clones. First, previous exposure of the immune system to the autoantigenic peptide by the DNA vaccine might have deviated the autoreactive MBP peptide-directed immune response to a T2 or T3 response. Potentially, no disease would evolve with such an immune response as evidenced in MHC congenic LEW rats (9). Second, DNA vaccination might have triggered a subencephalitogenic immune response, insufficient to cause disease. This response, however, might be sufficient to trigger a series of down-regulatory, antiidiotypic circuits specific for a particular MBP peptide. This scenario would be in line with that described for vaccination with attenuated encephalitogenic T cell lines and clones (10). Several lines of evidence presented here and elsewhere argue against the first and support the second possibility.

In this and our preceding studies, using the same protocols, we have analyzed the *ex vivo* MBP peptide-induced cytokine production and found no increase in production of T2 cytokines, e.g., IL-4 and IL-10. Instead, we found a global decrease in the antigen reactivity measured by IFN-γ production from draining lymph node cells (2). Moreover, we have shown that CpG DNA and a T1-type response are necessary for induction of protection in autoimmune conditions by DNA vaccination (11). Deliberate use of altered peptide ligands, thus implicating some form of degeneracy in the immune recognition, can lead to protection through T2-biased immunity (12). This was not the case here. Moreover, such an active immune deviation should have been able to protect against other encephalitogenic peptide stretches by bystander suppression (cross-tolerance) as in certain forms of oral tolerance (13, 14). Also, this was not the case in this study. Instead, the antigen specificity of suppression is consistent with either clonal anergy/deletion or an antiidiotypic protective immune response, as long as the encephalitogenic T cell repertoires differ after immunization with MBP_{GP}68–85 and MBP_{RAT}68–85. This, indeed, is the case, because we had shown that after immunization with MBP_{GP}63–88 compared with MBP_{RAT}63–88, differences in the T cell repertoire evolve with a predominant activation of $TCRBV8S2^+$ T cells and accumulation of these cells in the central nervous system (8). The difference between these peptides lies in a serine-to-threonine exchange, which represents a minor difference. Nevertheless, this conservative amino acid exchange resulted in dramatic differences in the repertoire. For the immune system this exchange cannot be considered as small, because one peptide represents a non-self-determinant, whereas the other is a self-determinant. MBP_{GP}68–85 is considered more immunogenic in rats compared with MBP_{RAT}68–85, but in equimolar high concentrations both peptides result in disease, which indicates that there is no lack of an encephalitogenic repertoire. Moreover, we have shown that the MBP_{GP}68–85-specific T cells induce EAE by cross-reactivity with self-MBP (8).

Together with our preceding work, our data reinforce that after immunization with self-MBP_{RAT}68–85 vs. non-self-MBP_{GP}68–85, differences in T cell repertoires evolve. Proliferative responses and numbers of IFN-γ-secreting cells thus differ greatly depending on the DNA vaccine used and the subsequent immunization with self- vs. non-self-MBP. Importantly, protection evolved only to the specific vaccination stretch. Furthermore, there were no T cell responses measurable against MBP89–101 or MOG37–54 after DNA vaccination with pZZ/MBP_{GP}68–85, pZZ/MBP_{RAT}68–85, or pZZ/MBP_{RAT}21.5 and subsequent immunization with MBP peptides MBP_{GP}68–85 or MBP_{RAT}68–85. This imposes that there is no cross-priming or antigen spreading (7) of myelin-reactive T cells by DNA vaccines and supports the high degree of specificity of the DNA vaccination approach. We thus failed to observe some general immunomodulatory capacities of DNA vaccines resulting in bystander suppression, as has been shown for altered peptide ligands (15). Also, in oral tolerance the situation differs: MBP_{GP}68–88 orally fed to Lewis rats resulted in protection from MBP_{GP}68–88- or MBP_{RAT}68–88-induced disease (14). This was not the case when MBP_{RAT}68–88 was fed, resulting in no protection against either peptide (14).

DNA vaccination with full-length MBP_{RAT}21.5 did not result in protection from EAE induced by either MBP_{GP}68–85 or MBP_{RAT}68–85. The reason could be that (*i*) the single-copy construct does not result in a high enough copy number of the potential encephalitogenic stretch to induce either T cell tolerance or a subencephalitogenic T cell response, (*ii*) potentially, a MHC class I-restricted T cell response is induced, which might not result in disease or protection, or (*iii*) there is no creation and presentation on MHC class II molecules of the encephalitogenic peptide stretch.

Our studies altogether suggest that a subencephalitogenic Th1 response can result in protection from EAE. This was supported by the finding that immune responses of spleen cells analyzed after DNA vaccination but before induction of EAE with peptides have shown a slight increase of peptide-reactive cells secreting IFN-γ rather than unresponsiveness (data not shown). Antigen-specific $CD4^+$ T cell responses are difficult to investigate on the clonal level, because they might persist only for a short time and the clonal expansions might be small (16). Use of tetrameric MHC–peptide complexes (17) and immunoscope analysis (18) will allow easier study of such responses in the future.

What are the actual molecular mechanisms dampening the encephalitogenic response? For example, the functional differentiation of the ensuing immune response with regard to expression of costimulatory molecules can be different after DNA vaccination (19). We have documented that DNA vaccination

indeed dampened the *ex vivo* peptide specific reactivity, but the mechanism is unclear. Furthermore, this dampening occurred irrespective of the success or failure of the vaccination, i.e., DNA vaccination with $MBP_{GP}68–85$ reduced the immune response against $MBP_{RAT}68–85$ despite no significant effect on the accumulated disease score. Thus, the cellular and/or molecular mediators responsible for protection remain unclear.

Our results indicate that before using DNA vaccines as potential therapeutic agents in humans, the exact T cell epitopes that drive disease may have to be known. The data also demonstrate that more knowledge about the actual mode of disease induction has to be gathered. Finally, our data imply that DNA vaccines for autoimmune conditions can be made without inducing unwanted immune alterations.

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Publikation 3

MHC Haplotype-dependent Regulation of MOG-induced EAE in Rats

Robert Weissert,* Erik Wallström,* Maria K. Storch,‡ Andreas Stefferl,§ Johnny Lorentzen,* Hans Lassmann,‡ Christopher Linington,§ and Tomas Olsson*

**Neuroimmunology Unit, Center of Molecular Medicine L8:04, Karolinska Hospital, S-17176 Stockholm, Sweden; ‡Neurological Institute, University of Vienna, A-1090 Vienna, Austria; and §Max-Planck-Institute for Neurobiology, D-82152 Martinsried, Germany*

Abstract

Experimental autoimmune encephalomyelitis (EAE) induced in the rat by active immunization with myelin-oligodendrocyte-glycoprotein (MOG) is mediated by synergy between MOG-specific T cells and demyelinating MOG-specific antibody responses. The resulting disease is chronic and displays demyelinating central nervous system (CNS) pathology that closely resembles multiple sclerosis. We analyzed major histocompatibility complex (MHC) haplotype influences on this disease. The MHC haplotype does not exert an all-or-none effect on disease susceptibility. Rather, it determines the degree of disease susceptibility, recruitment of MOG-specific immunocompetent cells, clinical course, and CNS pathology in a hierarchical and allele-specific manner. Major haplotype-specific effects on MOG-EAE map to the MHC class II gene region, but this effect is modified by other MHC genes. In addition, non-MHC genes directly influence both disease and T cell functions, such as the secretion of IFN-γ. Thus, in MOG-EAE, allelic MHC class II effects are graded, strongly modified by other MHC genes, and overcome by effects of non-MHC genes and environment. (*J. Clin. Invest.* 1998. 102:1265–1273.) Key words: MHC • EAE • myelin-oligodendrocyte-glycoprotein • interferon-γ • rat

Introduction

Human organ-specific inflammatory diseases such as multiple sclerosis (MS)[1] result from environmental influences interacting with multiple genes (1). The major histocompatibility complex (MHC) is well established as one of the gene regions predisposing to MS (2). However, the particular genes within the MHC that are involved and the mechanisms by which they act are still controversial (3). The MHC influence on the susceptibility to experimental autoimmune encephalomyelitis (EAE) induced by myelin basic protein (MBP), which is a purely T cell–dependent disease, has been studied extensively (4). In this model, certain MHC haplotypes are commonly described as disease permissive, while others are described as resistant. This can be considered a gross oversimplification of the situation in human organ-specific inflammatory diseases such as MS and type I diabetes, in which the autoimmune response involves both T cells and a variety of autoantibody responses (5–7). Such a more diverse autoimmune response may well initiate and/or include a large number of different immune effector mechanisms, which, in turn, are regulated by genes encoded both within and outside the MHC. This might modify the relative importance of specific MHC class II molecules in determining disease susceptibility.

We use myelin-oligodendrocyte-glycoprotein (MOG)-induced EAE in rats to study how differences in the MHC haplotypes influence a model that incorporates more of the complexity in immune effector mechanisms seen in human organ-specific inflammatory diseases. In MOG-EAE, a specific T cell response triggers an inflammatory cascade that opens the blood–brain barrier to circulating anti-MOG specific antibodies and serum complement. Antibody can then bind to the outer surface of the myelin sheath and mediate demyelination through a combination of complement and antibody-dependent cellular cytotoxicity-mediated mechanisms, while the local production of complement-derived pro-inflammatory factors amplifies the local inflammatory response. The histopathology of the lesions induced by this combination of immune effector mechanisms closely resembles that seen in MS (8, 9, the present work). To study the MHC in MOG-EAE on a constant non-MHC background and disease-inducing environment, we used MHC-congenic and intra-MHC recombinant congenic rats. The disease-inducing environmental challenge is titrated on two different MHC haplotypes. To investigate non-MHC gene modifying effects on the MHC, we varied the non-MHC background while holding the MHC haplotype and the disease-inducing environment constant. Clinical course, histopathology, and MOG-specific immunity are studied in relation to these manipulations.

Methods

Animals. Female rats 10–14 weeks of age were used in all experiments. All strains have been described (10) (see Table I). ACI rats were originally obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN); PVG-RT1[a] and BN rats from Harlan UK Limited (Blackthorn, UK); DA, DA.1H, LEW, LEW.1A, LEW.1AV1, and LEW.1W from the Zentralinstitut für Versuchstierzucht (Hannover, Germany); and LEW.1N, LEW.1AR1, LEW.1AR2, LEW.1WR1, and LEW.1WR2 from Prof. H. Hedrich (Medizinische Hochschule, Hannover, Germany). Rats were then locally bred in filter boxes and routinely tested for specific pathogens. Breeding pairs were checked for homozygosity by examination of a microsatellite marker located within the RT1 region. The experiments were approved by the local ethical committee.

Address correspondence to Dr. Robert Weissert, Department of Neurology, University of Tübingen, 72076 Tübingen, Germany. Phone: +49-7071-2982141; FAX: +49-7071-295260; E-mail: robert.weissert@uni-tuebingen.de

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1. *Abbreviations used in this paper:* CM, complete medium; CNS, central nervous system; ConA, concanavalin A; EAE, experimental autoimmune encephalomyelitis; LN, lymph nodes; MBP, myelin basic protein; MHC, major histocompatibility complex; MNC, mononuclear cells; MOG, myelin-oligodendrocyte glycoprotein; MS, multiple sclerosis; p.i., post immunization; RT, room temperature; RT1, MHC of rat; SI, stimulation index.

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Immunogens and Ags. The N-terminal sequences of rat MOG (amino acids 1–125) and rat S100β (11) were expressed in *Escherichia coli* and purified to homogeneity by chelate chromatography (12). The purified proteins in 6 M urea were then dialyzed against PBS to obtain preparations that were stored at −20°C. MOG and S100β were used in all cell cultures at 3 μg/ml. These Ag concentrations had given optimal stimulations in previous titration experiments. Concanavalin A (ConA) (Sigma Chemical Co., St. Louis, MO) was used at 1 μg/ml.

Induction and evaluation of EAE. Rats were anesthetized and injected intradermally at the base of the tail with 100 μl of inoculum containing 50 μg of rat MOG in saline emulsified (1:1) with CFA (Sigma Chemical Co., St. Louis, MO) containing 200 μg of *Mycobacterium tuberculosis* (strain H 37 RA; Difco Laboratories, Detroit, MI). For dose titration experiments in LEW.1A and LEW.1N rats, individual rats were injected with 1 μg, 5 μg, 10 μg, 20 μg, 50 μg, or 100 μg of MOG mixed with CFA as described above.

Rats were scored for clinical signs of EAE and were weighed daily up to 40 d post immunization (p.i.) by two alternating investigators. The signs were scored as follows: Grade 1, tail weakness or tail paralysis; and Grade 2, hind leg paraparesis or hemiparesis; Grade 3, hind leg paralysis or hemiparalysis; Grade 4, complete paralysis (tetraplegy), moribund state, or death. Ataxia was routinely assessed. A disease remission was defined as an improvement in disease score from either 3 or 4 to 1, or from 2, 3, or 4 to 0 that was maintained for at least 2 d consecutively. A relapse was defined as an increase in the clinical deficit of at least two points that lasted for at least two days.

Histopathological examination. Histological evaluation was performed on paraformaldehyde-fixed, paraffin-embedded sections of brains and spinal cords sampled at day 12 p.i. and day 40 p.i. Paraffin sections were stained with hematoxylin-eosin, Luxol fast blue, and Bielschowsky silver impregnation to assess inflammation, demyelination, and axonal pathology, respectively.

The inflammatory index was determined from the number of perivascular inflammatory infiltrates of each animal on an average of 15 complete cross sections of spinal cord. The degree of demyelination was evaluated separately for brain and spinal cord sections and semiquantitatively described and scored as follows: traces of perivascular or subpial demyelination (0.5); marked perivascular or subpial demyelination (1); confluent perivascular or subpial demyelination (2); massive confluent demyelination (e.g., half of spinal cord, one optic nerve complete) (3); and extensive demyelination (transverse myelitis, half of the cerebellar white matter or more, both optic nerves complete) (4).

In adjacent serial sections, immunohistochemistry was performed with Ab against the following targets: macrophages/activated microglia (ED1; Serotec, Oxford, UK), T cells (W3/13; Seralab, Sussex, UK), C9 (13), rat Ig (biotinylated α-rat; Amersham, Buckinghamshire, UK), and glial fibrillary acidic protein (GFAP; Boehringer Mannheim, Mannheim, Germany). Bound primary Ab was detected with a biotin–avidin technique previously described in detail (14). Control sections were incubated in the absence of primary Ab or with non-immune rabbit serum.

Fractionation and cultivation of mononuclear cells from LN and blood. Under deep anesthesia, draining inguinal (lymph nodes [LN]) were dissected out and put into DMEM (Life Technologies, Paisley, Scotland). LN were disrupted and mononuclear cells (MNC) washed twice in DMEM, resuspended in complete medium (CM) containing DMEM supplemented with 1% rat serum, 1% penicillin/streptomycin (Life Technologies), 1% glutamine (Life Technologies), and 50 μM 2-ME (Life Technologies), and flushed through a 70-μm plastic strainer (Falcon; Becton Dickinson–Vacutainer Systems, Franklin Lakes, CA). MNC were cultured at a concentration of 2×10^6 cells/ml in either 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark) with 100 μl of cell suspension per well, or 24-well flat-bottom plates (Falcon; Becton Dickinson–Vacutainer Systems) with 1000 μl of cell suspension per well at 37°C in a humidified atmosphere containing 5% CO_2.

Assays of Ag-induced proliferation and IFN-γ production. Proliferative experiments were performed in triplicates. 2×10^5 MNC/well in 100 μl of CM were cultured in 96-well round-bottom microtiter plates with or without Ag for 60 h and pulsed with 0.5 mCi [^{3}H]TdR (Amersham, Stockholm, Sweden) per well for 12 h. DNA was collected on glass fiber filters (Skatron Instruments Inc., Sterling, VA) and [^{3}H]TdR-incorporation was measured in a beta counter (Beckman Instruments Inc., Palo Alto, CA). To assess any T cell proliferation induced by minor bacterial contamination of the recombinant MOG, recombinant S100β was added to parallel T cell cultures. The MOG-specific proliferative stimulation index (SI) was obtained with the formula $1 + (SI_{MOG} - SI_{S100\beta})$.

Ag-induced IFN-γ mRNA expression was measured by culture of 2×10^6 MNC in 1000 μl of CM per well in 24-well plates in the presence and absence of MOG, S100β, and ConA for 48 h at 37°C and 5% CO_2. The cells were collected, washed twice in PBS (Life Technologies), and counted, and 1×10^5 cells were dried onto restricted areas of glass slides (Probe On; Fisher Scientific Co., Pittsburgh, PA) for each Ag. In situ hybridization and counting of positive cells were performed as described (15). Responses induced by bacterial contamination of the recombinant MOG were controlled by using recombinant S100β protein. The numbers of IFN-γ mRNA-expressing cells induced by recombinant S100β were 0–4 per 1×10^5 cells. The MOG-specific numbers of IFN-γ mRNA-expressing cells were expressed as MOG minus S100β IFN-γ mRNA-expressing cells.

An Elispot method was used to enumerate T cells secreting IFN-γ after Ag exposure (15). Nitrocellulose-bottom 96-well plates (MAHA; Millipore, Molsheim, France) were coated with the mAb DB1 (anti–rat IFN-γ; a generous gift of Dr. Peter van der Meide, TNO Primate Centre, Rijswijk, The Netherlands). After blocking with DMEM containing 5% FCS (Life Technologies), we added triplicates of 4×10^5 cells in 200 μl of CM per well and antigen to the plates and incubated them for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. Secreted and bound IFN-γ was visualized with biotinylated mDB12 (anti-rat IFN-γ; Dr. Peter van der Meide), avidin–biotin peroxidase (Vector Laboratories Inc., Burlingame, CA), and staining with carbazole (Sigma Chemical Co.). In control cultures, S100β induced 0–1 cell per 4×10^5 cells of IFN-γ–secreting cells. The MOG-specific numbers of IFN-γ–secreting cells were expressed as MOG minus S100β IFN-γ–secreting cells.

Enumeration of cells secreting Ag-specific antibodies. An Elispot method was used for enumeration of plasma cells secreting anti-MOG specific antibodies (16). Nitrocellulose-bottom 96-well plates (MAHA; Millipore) were coated for 12 h with either 3 μg/ml MOG in PBS, 3 μg/ml S100β in PBS, or PBS alone. The plates were then washed with PBS and blocked with DMEM containing 5% FCS (Life Technologies). Triplicates of 2×10^5 MNC per well in 100 μl of CM for each antigen were incubated for 16 h at 37°C in a humidified atmosphere containing 5% CO_2. Secreted and bound antibodies were visualized by polyclonal rabbit anti–rat Ig (Dako, Glostrup, Denmark), biotinylated anti–rabbit Ig (Dako) followed by avidin–biotin peroxidase (Vector Laboratories), and staining with carbazole (Sigma Chemical Co.). The number of S100β-specific spots was equal to the number of spots in PBS-coated wells. The mean number of spots in PBS-coated control wells was subtracted from the number of spots recorded in the MOG-coated wells. The results are expressed as the number of anti-MOG Ab-secreting cells per 10^5 MNC.

Collection of serum samples and ELISA. Blood samples for Ab measurements were taken at days 12 p.i. and 40 p.i. 96-well ELISA plates (Nunc) were coated with 2.5 μg/ml (100 μl/well) recombinant rat MOG overnight at 4°C. Plates were washed with PBS/0.05% Tween and blocked for 1 h at room temperature (RT). Diluted serum samples were added after washing and plates were incubated for 1 h at RT. Then, plates were washed, and diluted rabbit–anti-rat antiserum (Nordic, Tilburg, The Netherlands) was added and incubated for 1 h at RT. Unbound antibodies were then removed by washing prior to the addition of peroxidase-conjugated goat–anti-rabbit antiserum (Nordic) diluted in PBS/0.05% Tween (1:10,000). After 30 min, incu-

bation plates were washed thoroughly, and bound antibodies were visualized through addition of 3,3′,5′5′-tetramethylbenzidine (TMB; Sigma Chemical Co.). The enzymatic reaction was stopped with 1 M HCl after 15 min of incubation in darkness, and the optical density was read at 450 nm.

Statistics. Differences in the occurrence of inflammation and demyelination between MOG-immunized strains were tested for significance with Fischer's exact test. T and B cell responses were tested for significance by comparing responses in MOG-immunized rats with responses in CFA-immunized rats, using Student's *t* test for variables with normal distribution and the Mann-Whitney U test for other variables. Normality testing was performed with the Shapiro-Wilk test. Corrections for multiple comparisons were made with the Bonferroni method.

Results

Modulation of MOG-EAE by RT1 haplotypes on a constant non-MHC LEW background. We examined how different RT1 haplotypes in combination with LEW non-MHC genes affected clinical and histopathological disease and the MOG-specific immune response. For this purpose, we used a series of MHC congenic LEW rat strains ($RT1^n$, $RT1^a$, $RT1^{av1}$, $RT1^u$, and $RT1^l$) selected to provide the greatest possible variety of MHC class II alleles (17).

Disease outcome in these LEW RT1 congenic rat strains ranked from mild or no disease to acute lethal disease (Fig. 1 *A*). The most fulminant disease course was seen in LEW.1N ($RT1^n$) rats, which developed early onset acute lethal disease with paraplegia, ataxia, and extensive demyelinating plaques. LEW.1A ($RT1^a$) and LEW.1AV1 ($RT1^{av1}$) rats showed a chronic and/or relapsing type of disease. Some LEW.1W ($RT1^u$) rats displayed clinical signs at a late time point, while LEW ($RT1^l$) rats remained free from clinical signs.

The intensity of the histopathological lesions paralleled the severity of clinical signs (Fig. 2, see Fig. 5). The hyperacute fatal disease course in LEW.1N rats was associated with widespread inflammatory demyelinating lesions in the CNS at day 12 p.i. These actively demyelinating lesions contained large numbers of polymorphonuclear cells, as well as T cells and macrophages, and were associated with extensive deposition of IgG and C9. LEW.1AV1 and LEW.1A rats exhibited a mixture of purely inflammatory and mixed inflammatory-demyelinating lesions on day 12 p.i., which by day 40 p.i. had progressed to form large areas of confluent demyelination. Only one out of thirteen LEW.1W rats was found to have a low degree of inflammatory pathology in the CNS on day 12 p.i., although by day 40 p.i., several rats exhibited a subclinical inflammatory response in the CNS that was in some cases accompanied by minor perivascular demyelination. However, the two most severely diseased LEW.1W rats were found to have large confluent focal demyelinating lesions in the spinal cord, medulla oblongata, and cerebellum. In accordance with the lack of clinical disease in LEW rats, no CNS pathology was observed on day 12 p.i. and only a small number of rats exhibited moderate perivascular inflammation in the spinal cord on day 40 p.i.

We studied if the RT1 haplotype-regulated disease outcome was correlated to any qualitative or quantitative aspect of the MOG-specific immune response on day 12 p.i. (Fig. 3 *A*). MOG-induced T cell proliferative responses and the number of IFN-γ mRNA-expressing cells in MNC from the draining LN correlated with the severity of clinical disease. When compared with CFA-injected controls, LEW.1N rats displayed the highest stimulation indices, followed by LEW.1A, LEW.1W, and LEW rats. A similar hierarchy was apparent for the number of MOG-induced IFN-γ mRNA-expressing cells with the highest numbers in the LEW.1N strain, followed by LEW.1A, LEW.1W, and LEW rats. All strains showed similarly high proliferation indices and high numbers of IFN-γ mRNA-expressing cells after mitogenic stimulation with ConA (data not shown). The degree of the MOG-specific B cell response was also similarly ranked in that the number of anti-MOG Ab secreting cells was high in LEW.1N and LEW.1A, while LEW.1W and LEW had low numbers. This was paralleled by the anti-MOG Ab titers. Thus, the onset of clinical disease and lesion growth/activity in LEW RT1 congenic rat strains corre-

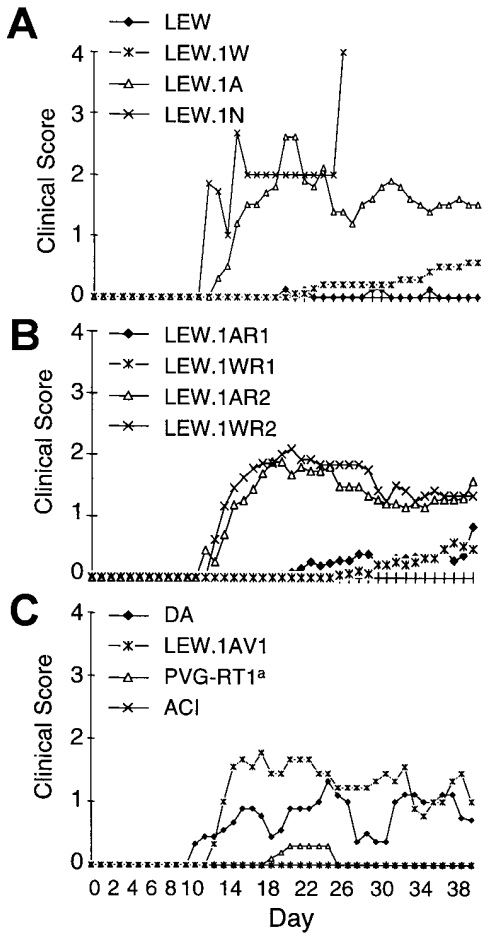

Figure 1. Clinical course of MOG-induced EAE in MHC congenic and intra-MHC recombinant congenic rat strains. Mean daily scores of (*A*) LEW ($n = 8$), LEW.1W ($n = 14$), LEW.1A ($n = 10$), and LEW.1N ($n = 14$); (*B*) LEW.1AR1 ($n = 27$), LEW.1WR1 ($n = 28$), LEW.1AR2 ($n = 18$), and LEW.1WR2 ($n = 13$); (*C*) DA ($n = 9$), LEW.1AV1 ($n = 10$), PVG-$RT1^a$ ($n = 10$), and ACI ($n = 13$) rats. LEW.1N rats developed an acute lethal disease with ataxia and paraplegia (13 of 14 rats died between days 12–15 p.i.). LEW.1A, LEW.1AR2, and LEW.1WR2 rats had chronic disease, while DA and LEW.1AV1 rats had chronic and/or relapsing disease. LEW.1W, LEW.1AR1, LEW.1WR1, and PVG-$RT1^a$ rats were semiprotected. LEW and ACI rats showed no clinical signs of disease during the 40 d observation period.

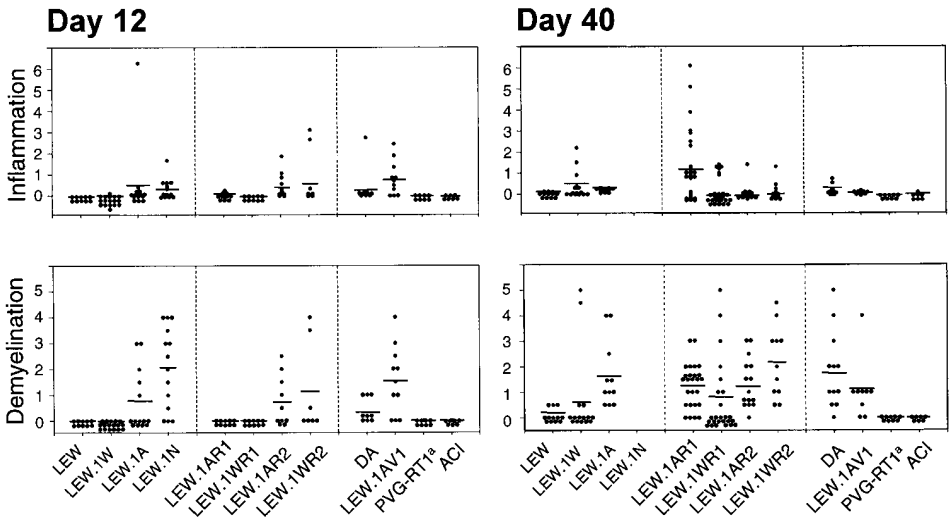

Figure 2. Semiquantitative assessment of CNS inflammation and demyelination. Individual rats sacrificed on day 12 p.i. or day 40 p.i. are represented by black dots and group means by horizontal lines. At day 12 p.i., inflammation was more frequent in LEW.1A ($P = 0.002$), LEW.1AR2 ($P < 0.001$), LEW.1WR2 ($P = 0.003$), and LEW.1N ($P < 0.001$) rats compared with LEW.1W rats, and demyelination was more frequent in LEW.1A ($P = 0.048$), LEW.1AR2 ($P = 0.012$), and LEW.1N ($P < 0.001$) rats than in LEW.1W rats. At day 40 p.i., no differences remained in regard to inflammation in rats with the LEW background ($P > 0.05$), but demyelination was more frequent in LEW.1A ($P = 0.001$), LEW.1AR1 ($P = 0.003$), LEW.1AR2 ($P = 0.03$), and LEW.1WR2 ($P = 0.001$) rats compared with LEW.1W rats. When comparing rats sharing the RT1^{av1} haplotype but having different non-MHC background genomes, we found that inflammation and demyelination were less frequent in PVG-RT1^a and ACI rats at day 12 p.i. (inflammation, $P = 0.010$ and 0.012; demyelination, $P = 0.010$ and 0.012) and day 40 p.i. (inflammation, $P = 0.001$ and 0.026; demyelination, $P = 0.002$ and 0.007), but not in DA rats ($P > 0.05$ for all variables and timepoints), compared with LEW.1AV1 rats. All P values were calculated with Fischer's exact test corrected for multiple comparisons with the Bonferroni method.

lated to the degree of functional activation and expansion of MOG-specific T and B cells.

RT1 determines the Ag concentration threshold necessary to trigger disease. The effect of the RT1 haplotype on determining sensitivity to disease induction (eg. environment) was investigated in the highly susceptible MHC congenic strains LEW.1N and LEW.1A. Animals were immunized with doses of MOG ranging from 1 μg to 100 μg MOG. LEW.1N rats developed both clinical disease and CNS pathology at all Ag doses tested, whereas LEW.1A rats only developed disease when injected with ≥ 50 μg of MOG (Fig. 4, *A* and *B*). Interestingly, not only disease severity but also disease course were dose related, with the majority of LEW.1N rats developing a chronic, rather than acute, disease when injected with lower

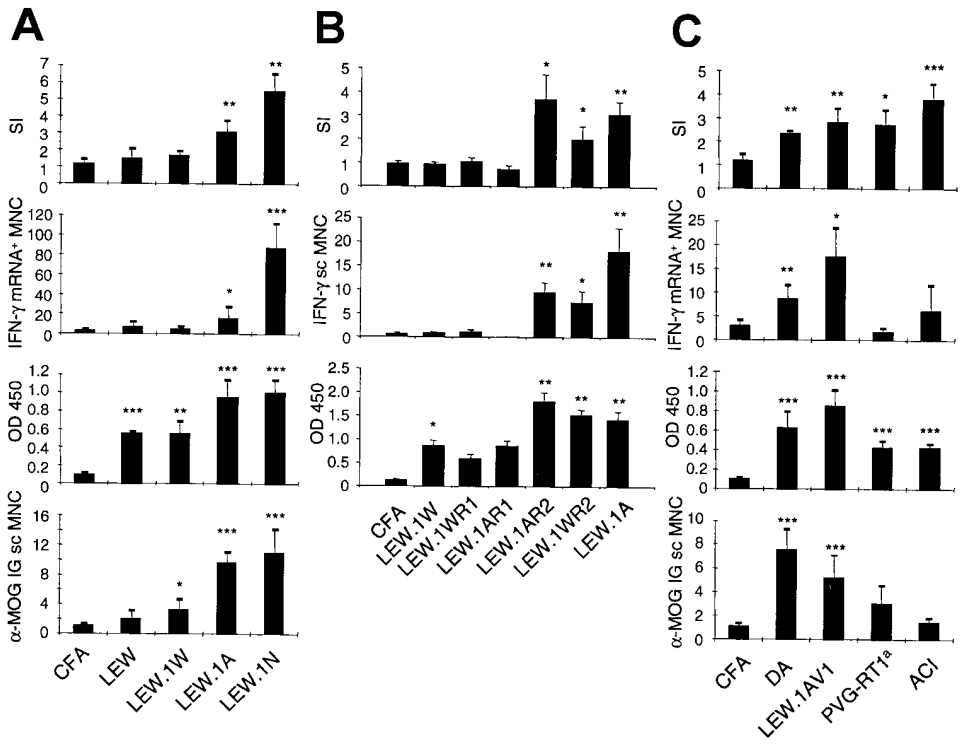

Figure 3. MOG-specific T and B cell responses at day 12 p.i. (*A*) MOG-immunized LEW ($n = 8$), LEW.1W ($n = 8$), LEW.1A ($n = 8$), LEW.1N ($n = 8$) rats, and CFA-immunized rats ($n = 10$); (*B*) MOG-immunized LEW.1W ($n = 6$), LEW.1AR1 ($n = 5$), LEW.1WR1 ($n = 6$), LEW.1A ($n = 5$), LEW.1AR2 ($n = 5$), LEW.1WR2 ($n = 6$), and CFA-immunized rats ($n = 6$); (*C*) MOG-immunized DA ($n = 9$), LEW.1AV1 ($n = 6$), PVG-RT1^a ($n = 8$), ACI ($n = 7$) rats, and CFA-immunized rats ($n = 10$). SI, proliferative T cell responses from draining LN; IFN-γ mRNA$^+$ MNC, numbers of Ag-specific IFN-γ mRNA-expressing LN cells; IFN-γ sc MNC, numbers of Ag-specific IFN-γ–secreting LN cells; OD 450, specific anti-MOG IgG levels in serum; α-MOG IG sc MNC, specific anti-MOG Ab-secreting cells per 10^5 LN cells. Each bar represents mean±SEM. Stars indicate significance of responses in MOG-immunized rats compared with CFA-immunized rats (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). After normality testing with the Shapiro-Wilks test, P values were calculated with the Student's t test in cases of normal distribution, otherwise the Mann-Whitney U test was used.

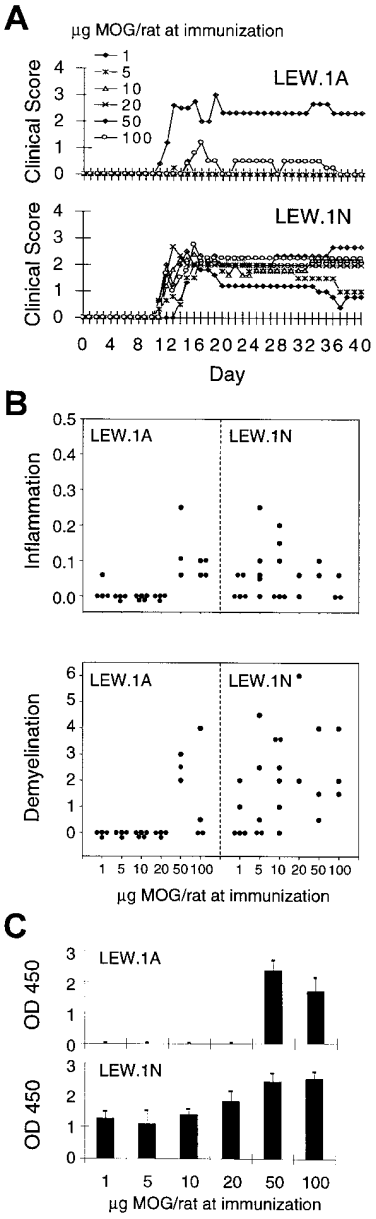

Figure 4. Titration of Ag for disease triggering in LEW.1A and LEW.1N rats. Groups of 5–6 LEW.1N and 4–5 LEW.1A rats were immunized with 1, 5, 10, 20, 50, or 100 μg of MOG in CFA. (*A*) Mean daily clinical scores (in the LEW.1N groups immunized with 100, 50, and 20 μg of MOG 2 of 5, 4 of 6, and 4 of 6 rats died between days 12–14 p.i., respectively, and in the LEW.1A groups immunized with 100 and 50 μg MOG, 1 of 5 and 2 of 5 rats died between days 13–17 p.i., respectively); (*B*) CNS inflammation and demyelination on day 40 p.i.; (*C*) MOG-specific serum Ab titers day 12 p.i. LEW.1N rats developed disease at all tested Ag doses, while LEW.1A rats developed disease only at doses > 20 μg of MOG. Disease was paralleled by MOG-specific serum Ab.

doses of autoantigen. Once again, the induction of clinical disease correlated with the MOG-specific immune response (Fig. 4 *C*). LEW.1N rats displayed high MOG-specific Ab titers at all doses tested, while LEW.1A rats raised MOG-specific Ab only at doses of 50 or 100 μg. Thus, the identity of the MHC also determined the amount of Ag necessary to trigger autoimmune disease.

RT1.B/D allelic products are the key regulators of susceptibility in MOG-EAE. To narrow the regions within the MHC that modulated the disease phenotypes, we used different combinations of RT1.A/B/D/C alleles of the $RT1^a$ and $RT1^u$ haplotype on the permissive LEW background (10, 15) (Table I). Intra-MHC recombinant rats with recombination events between RT1.A and RT1.B (LEW.1AR1 and LEW.1WR2, respectively) and RT1.D and RT1.C (LEW.1AR2 and LEW.1WR1, respectively) allowed us to map the region within the MHC and determine susceptibility to MOG-induced EAE. The clinical disease courses are outlined in Fig. 1 *B*, and the histopathological evaluation is given in Fig. 2.

Table I. Inbred Rat Strain Designations with Fine Mapping and Recombination Sites of the Rat MHC (RT1)

Strain	Haplotype	Class I RT1.A	Class II RT1.B	Class II RT1.D	Class III	Class I RT1.C
DA	av1	a	a	a	av1	av1
ACI	av1	a	a	a	av1	av1
PVG-$RT1^a$ (DA)	av1	a	a	a	av1	av1
LEW.1AV1 (DA)	av1	a	a	a	av1	av1
LEW.1A (AVN)	a	a	a	a	a	a
LEW.1N (BN)	n	n	n	n	n	n
LEW.1W (WP)	u	u	u	u	u	u
LEW	l	l	l	l	l	l
LEW.1AR1	r2	a	u	u	u	u
LEW.1AR2	r3	a	a	a	u	u
LEW.1WR1	r4	u	u	u	a	a
LEW.1WR2	r6	u	a	a	a	a
DA.1H	h	h	n	n	?	?
BN	n	n	n	n	n	n

Donor strain in parentheses.

Most LEW.1AR2 ($RT1^{r3}$) rats that are $RT1.A^a/B^a/D^a$ and $RT1.C^u$ (17/18) and most LEW.1WR2 ($RT1^{r6}$) rats that are $RT1.A^u$ and $RT1.B^a/C^a/D^a$ (12/13) developed early disease, sharing the MHC class II $RT1.B^a/D^a$ alleles of the LEW.1A rat (Table I). Only a low number of LEW.1AR1 ($RT1^{r2}$) rats that are $RT1.A^a$ and $RT1.B^u/D^u/C^u$ developed clinical disease (7/27). This was also the case for LEW.1WR1 ($RT1^{r4}$) rats with $RT1.A^u/B^u/D^u$ and $RT1.C^a$ (7/28). Both strains share the $RT1.B^u/D^u$ alleles of the LEW.1W rat in the MHC class II region.

Consistent with the early onset of clinical disease in LEW.1AR2 rats, at day 12 p.i., two-thirds of the rats had inflammation alone or inflammation associated with demyelination, while by day 40 p.i., most animals had demyelinating lesions in the spinal cord and the brain. A number of LEW.1WR2 rats showed inflammation on day 12 p.i. that was not associated with demyelination, but large confluent plaques of demyelination were observed by day 40 p.i., predominantly in the spinal cord (Fig. 5). LEW.1WR1 and LEW.1AR1 rats had no or minimal inflammation and no demyelination in the CNS by day 12 p.i. As with the LEW.1W strain, some rats from these two strains developed demyelinated lesions and clinical symptoms late in the observation period. The LEW.1AR1 strain was especially prone to develop CNS lesions, as discussed below.

T cell proliferation assays, Elispot assays to assess the number of IFN-γ–secreting cells, and serum titers of anti-MOG Ab on day 12 p.i. revealed that rats having the $RT1.B^a/D^a$ alleles (LEW.1A, LEW.1AR2, and LEW.1WR2) had higher MOG-specific immune responses than rats having the $RT1.B^u/D^u$ alleles (LEW.1W, LEW.1AR1, and LEW.1WR1) (Fig. 3 *B*). All strains had high proliferation indices and between 500 and 2,000 IFN-γ–secreting cells per 4×10^5 cells after stimulation with ConA.

The main susceptibility locus within the MHC for MOG-induced EAE thus maps to the RT1.B/D region. This was

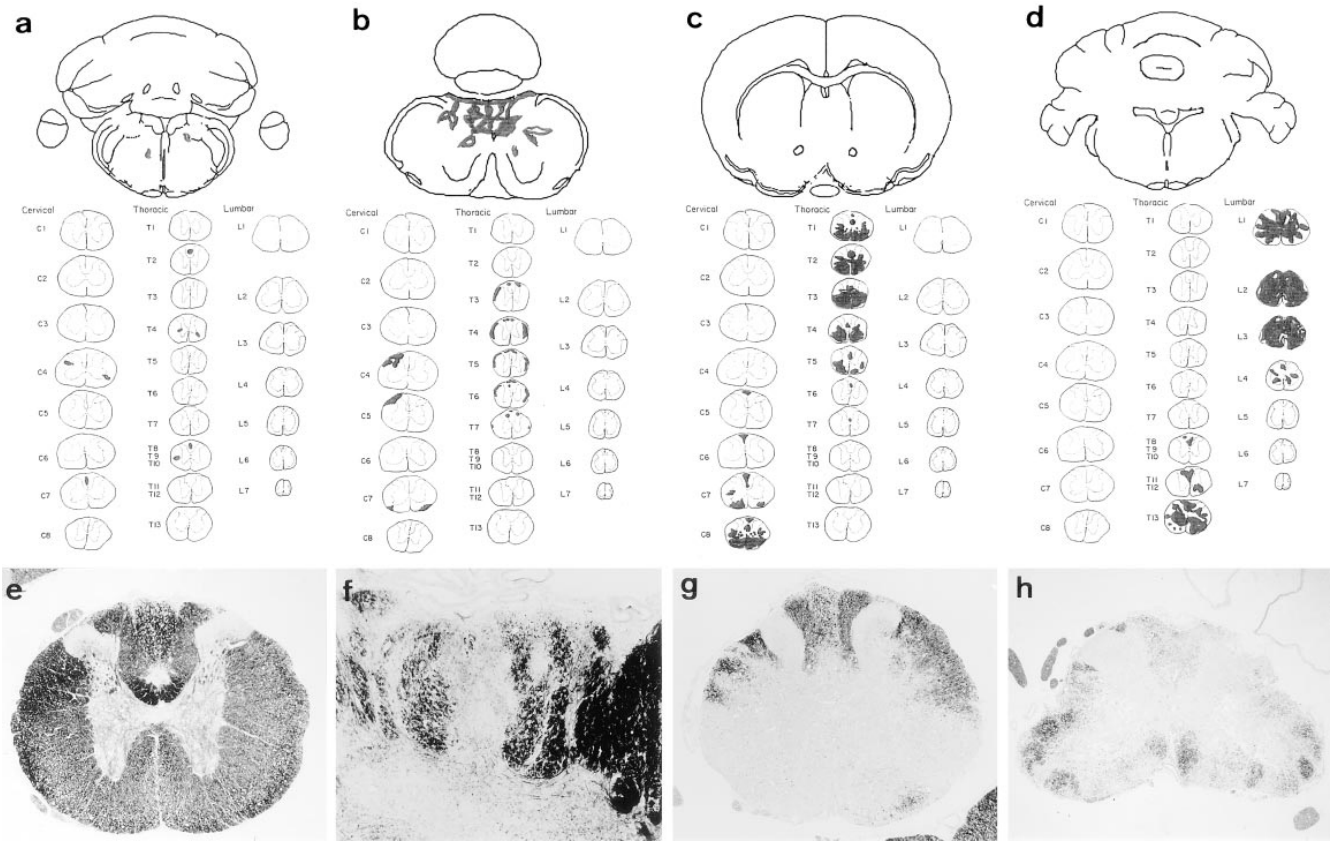

Figure 5. Histopathology of different RT1 congenic rat strains after immunization with MOG. Comparison of lesional topography and lesion formation of actively induced MOG-EAE is illustrated for LEW (*a*, *e*), LEW.1N (*b*, *f*), LEW.1A (*c*, *g*), and LEW.1WR2 (*d*, *h*) rats. *e–h* illustrate the degree of demyelination (Luxol fast blue myelin stain). *a*, *e*: LEW rats show small perivascular rims of demyelination ($e \times 27$). *b*, *f*: LEW.1N rats exhibit widespread demyelination oriented to the CSF and in association with the area postrema ($f \times 70$). *c*, *g*: LEW.1A rats show focal confluent demyelination in the spinal cord ($g \times 27$). *d*, *h*: LEW.1WR2 rats frequently present transverse myelitis ($h \times 25$).

strengthened by the observation that four out of four investigated DA.1H ($RT1^h$) rats, which carry the $RT1.B^n/D^n$ alleles in the MHC class II but different MHC class I and III alleles (Table I), developed an acute and lethal disease similar to the LEW.1N rat strain (data not shown). Further support comes from the observation that BN rats, which are commonly considered to be resistant to EAE-induction (18) but which are also $RT1.B^n/D^n$ (Table I), are highly susceptible to MOG-induced EAE (Linington, C., Weissert, R., unpublished observation). To rule out possible influences from genetic drift of the LEW.1N strain, intercross F2(LEW.1N × LEW) rats were investigated to confirm linkage of acute lethal disease to $RT1^n$ (data not shown).

An influence of the RT1.A region (MHC class I) on lesion development in MOG-induced EAE was suggested by the more pronounced demyelination in the brain and spinal cord in the LEW.1AR1 ($RT1^{r2}$) compared with the LEW.1W ($RT1^u$) strain on day 40 p.i. (Fig. 2). Due to the recombination between $RT1.A^a$ and $RT1.B^u/D^u/C^u$ ($RT1^{r2}$) in the LEW.1AR1 strain (Table I), this difference could map to $RT1.A^a$ or genes downstream within the MHC congene fragment, or an interaction between $RT1.A^a$ and $RT1.B^u/D^u/C^u$, as discussed below. Additional disease-modifying influences mediated by RT1.C, MHC class III region genes, or loci located further downstream were suggested by the comparison of the disease course between the LEW.1AV1 and LEW.1A rats, which differ in the RT1 downstream of the RT1.D gene region (Table I). While none out of ten LEW.1A rats had a relapsing/remitting type of disease, but instead developed chronic neurological deficits, five out of ten LEW.1AV1 rats developed a relapsing remitting disease course ($P = 0.02$; Fisher's exact test).

Modulation of $RT1^{av1}$ influences by four different non-MHC backgrounds. We investigated the degree of susceptibility, clinical disease course, and lesional pathology in four different $RT1^{av1}$ haplotype-bearing rat strains: DA, LEW.1AV1, PVG-$RT1^a$, and ACI (Figs. 1 *C* and 2). Five out of nine DA rats and five out of ten LEW.1AV1 rats exhibited a relapsing/remitting disease course with classical ascending paraparesis. In addition, two out of ten LEW.1AV1 rats displayed ataxia (data not shown). Thus, the $RT1^{av1}$ haplotype permitted a similar clinical disease phenotype on the LEW and DA rat non-MHC genes, with slightly more acute symptoms in the LEW.1AV1 rat. In contrast, the PVG and ACI non-MHC genes were less permissive for MOG-induced EAE in conjunction with the $RT1^{av1}$ haplotype.

At day 12 p.i., PVG-$RT1^a$ and ACI rats displayed little or no CNS pathology. In contrast, LEW.1AV1 and DA rats had already developed extensive CNS lesions by day 12 p.i. In both strains, large plaques of demyelination with variable demyelinating activity were found in both the brain and spinal cord by day 40 p.i. Actively demyelinating areas were characterized by infiltration with T cells and macrophages, and destruction of

myelin was associated with the deposition of C9 and IgG. In inactive areas, the inflammatory lesions contained numerous macrophages. Gliotic scar formation was evident in areas with confluent demyelination and axonal density was only slightly reduced. Thus, the $RT1^{av1}$ haplotype on two different EAE-permissive non-MHC backgrounds resulted in similar histopathological changes that were strongly reminiscent of those seen in MS.

To study if the regulation of disease outcome by interactions involving the MHC haplotype and non-MHC genes was related to any qualitative or quantitative feature of the autoimmune response to MOG, we analyzed MOG-specific T and B cell responses in the four strains with the $RT1^{av1}$ haplotype on day 12 p.i. (Fig. 3 *C*). MOG induced a proliferative response in the LN cell cultures of all four rat strains on day 12 p.i. However, an increase in numbers of IFN-γ mRNA-expressing cells was recorded only in the disease-susceptible LEW.1AV1 and DA rat strains (Fig. 3 *C*), although all four strains displayed similar increases after mitogenic stimulation with Con A (data not shown). The numbers of anti-MOG Ab-secreting cells were also higher in DA rats and LEW.1AV1 rats than in PVG-$RT1^a$ rats and ACI rats. This correlated with the anti-MOG Ab titers. Thus, the $RT1^{av1}$ haplotype is permissive for induction of a MOG-specific autoimmune response in all strains, but the functional maturation of this response is strongly influenced by non-MHC genes.

Discussion

We demonstrate that MHC haplotypes differ in the severity of the ensuing disease they permit in response to a constant autoantigenic MOG challenge. Furthermore, the MHC haplotype determines the amount of autoantigen needed to induce disease. Thus, MHC haplotypes are not strictly disease permissive or resistant. Instead, there is a hierarchy in MHC haplotype regulation of disease, with high responder haplotypes such as $RT1^n$ and $RT1^h$, intermediate ones such as $RT1^a$, $RT1^{av1}$, $RT1^{r3}$, and $RT1^{r6}$, and low responders such as $RT1^u$, $RT1^{r2}$, $RT1^{r4}$, and $RT1^l$. Notably, no single haplotype is completely protected (19). This form of MHC haplotype-regulated graded influence on MOG-EAE, a model that is more like MS than any other EAE model (8, 9, the present work), gives perspective on how HLA haplotypes might be involved in the regulation of human MS. Similar to what is observed here, graded influences are apparent. The HLA-DR2(15) haplotype strikingly increases the risk to develop MS, followed by HLA-DR3(17), while a series of other HLA haplotypes also permit disease (20). Hypothetically, and consistent with the present observations in MOG-EAE, the former haplotypes would allow disease with milder environmental triggers and/or a less susceptible non-MHC background genome, and vice versa, with regard to less permissive HLA haplotypes. Since neither the triggering events nor the background genomes can be experimentally manipulated in humans, we believe that our findings in MOG-induced EAE are important as a basis for further mechanistic studies to understand how the MHC regulates complex organ-specific inflammatory diseases. At present, we hypothesize that allelic variations in the MHC class II molecule peptide binding abilities might be responsible for the dramatic differences between the different haplotypes in response to immunization with MOG. The antigen–MHC interaction decisively influences the developing T cell repertoire within the thymus (21–24) and the degree and quality of peripheral T cell activation (25, 26). T cell transfer experiments show that the numbers and functional differentiation of adoptively transferred T cells correlate with the severity of the subsequent disease (27–29). We currently explore how allelic variations in the MHC class II molecule binding abilities to MOG peptides correlate to the selection, expansion, and epitope specificity of encephalitogenic T and B cells. This study strongly suggests that the mechanism for MHC haplotype regulation of the degree of ensuing disease is by regulation of the quantity and quality of the autoimmune response after autoantigenic challenge, since these factors directly correlated to each other.

The use of intra-MHC recombinant rats mapped major regulatory influences to the MHC class II region. A strong argument in favor of a role for RT1.B and/or RT1.D molecules (equivalent to human HLA-DQ and HLA-DR, respectively) is the different MHC haplotype restriction of MOG-induced disease (19, 30, the present work) as compared with MBP-induced EAE in LEW compared with LEW.1N rats (31, Table II). This is difficult to reconcile with any general immunoregulatory genes in the MHC class II region, since only allelic forms of the RT.1B and RT.1D molecules would discriminate between Ags. Interestingly, the $RT1^{av1}$ haplotype is highly disease permissive to MBP, PLP, and MOG as well (32–34, the present work). By this ability, $RT1^{av1}$ is CNS-autoantigen promiscuous, unlike the $RT1^n$ haplotype. Since MHC class II molecules are restriction elements for $CD4^+$ T cells, another argument supporting the major role of the RT1.B and RT1.D is the correlation between the severity of clinical disease and the magnitude of the MOG-specific T cell response. MOG-specific B cell responses also show a correlation to disease severity, a phenomenon that we regard as probably secondary to the T cell response (35–37). The degree of the ensuing B cell response might well be causally related to the degree of ensuing disease in view of the importance of anti-MOG antibodies in demyelination and the potential antigen-presenting ability of B cells (38). Apart from the classical MHC class II molecules themselves, some other immunoregulatory genes also map within the MHC class II region. An important example of this is RT1.DM (the equivalent of the human HLA-DM) (39), and a final proof for a particular gene being instrumental may necessitate its knockout and/or transgenic expression. Furthermore, there are haplotype-specific disease-promoting influences from genes down- or upstream from the MHC class II region. Recombinations between the MHC class I and class II region showed that the $RT1.A^a$ allele in conjunction with the $RT1.B^u/D^u$ ($RT1^{r2}$) alleles resulted in conspicuous demyelination late in disease compared with rats with $RT1.A^u/B^u/D^u$

Table II. Autoantigen Restrictions in Rat EAE

	RT1 Haplotype			
Autoantigen	$RT1^l$	$RT1^u$	$RT1^a/RT1^{av1}$	$RT1^n$
MBP	+++ (31)	ND	+++ (32, 34)	− (31)
PLP	+ (33)	ND	+++ (34)	ND
MOG	(+)* (19, 30)	+*	+++*	++++*

*The present work.

($RT1^u$) alleles. The mechanism responsible for this effect is unclear and is under study in our laboratories. Besides effects from the MHC class I region itself, this might depend on the TAP2 genes that exhibit allelic polymorphisms between LEW.1W ($TAP2^b$), LEW.1A ($TAP2^a$), and LEW.1AR1 ($TAP2^b$) rats, which results in a differential loading of peptides onto class I MHC molecules (cim effect) (40). Genes upstream from the MHC class II region in the $RT1^{av1}$ haplotype might also modulate clinical disease type since LEW.1AV1, but not LEW.1A, rats developed a relapsing/remitting disease course.

Non-MHC genes could abrogate MHC-permitted susceptibility to MOG-induced EAE. By changing non-MHC genes on a constant MHC haplotype, we show that non-MHC genes are disease protective in PVG-$RT1^a$ and ACI, and permissive in DA and LEW.AV1 rats. Interestingly, while all strains mounted a marked proliferative response to MOG in vitro, only the disease-permissive ones displayed MOG-induced IFN-γ mRNA expression and a strong MOG-specific B cell response. This demonstrates that genes outside the MHC determine the pathogenic potential of T cells by regulating their functional differentiation, for instance, at the level of IFN-γ expression and secretion, or providing B cell help. Findings consistent with this have been reported in mice (41). Furthermore, in MBP-induced EAE, exogenous IL-12 was necessary for disease induction in B10.S mice (42). We are currently mapping the non-MHC genes that modulate RT1 mediated effects in MOG-EAE by genome-wide microsatellite screening (43) of an F2 intercross between DA and ACI rats.

In conclusion, we have explored the immunogenetic characteristics of the new and very MS-like MOG-EAE in rats. This gives an experimental basis for a hypothetical model of MHC influences in complex organ-specific inflammatory diseases, in which an MHC-haplotype–dependent hierarchically ordered susceptibility is modulated by environmental triggers and other, still undefined, non-MHC background genes.

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Publikation 4

MHC Class II-Regulated Central Nervous System Autoaggression and T Cell Responses in Peripheral Lymphoid Tissues Are Dissociated in Myelin Oligodendrocyte Glycoprotein-Induced Experimental Autoimmune Encephalomyelitis[1]

Robert Weissert,[2,3]*† Katrien L. de Graaf,[3]*† Maria K. Storch,‡ Silvia Barth,* Christopher Linington,§ Hans Lassmann,‡ and Tomas Olsson†

We dissected the requirements for disease induction of myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis in MHC (RT1 in rat) congenic rats with overlapping MOG peptides. Immunodominance with regard to peptide-specific T cell responses was purely MHC class II dependent, varied between different MHC haplotypes, and was linked to encephalitogenicity only in RT1.B^a/D^a rats. Peptides derived from the MOG sequence 91–114 were able to induce overt clinical signs of disease accompanied by demyelinated CNS lesions in the RT1.B^a/D^a and RT1^n haplotypes. Notably, there was no detectable T cell response against this encephalitogenic MOG sequence in the RT1^n haplotype in peripheral lymphoid tissue. However, CNS-infiltrating lymphoid cells displayed high IFN-γ, TNF-α, and IL-4 mRNA expression suggesting a localization of peptide-specific reactivated T cells in this compartment. Despite the presence of MOG-specific T and B cell responses, no disease could be induced in resistant RT1^l and RT1^u haplotypes. Comparison of the number of different MOG peptides binding to MHC class II molecules from the different RT1 haplotypes suggested that susceptibility to MOG-experimental autoimmune encephalomyelitis correlated with promiscuous peptide binding to RT1.B and RT1.D molecules. This may suggest possibilities for a broader repertoire of peptide-specific T cells to participate in disease induction. We demonstrate a powerful MHC class II regulation of autoaggression in which MHC class II peptide binding and peripheral T cell immunodominance fail to predict autoantigenic peptides relevant for an autoaggressive response. Instead, target organ responses may be decisive and should be further explored. *The Journal of Immunology,* **2001, 166: 7588–7599.**

Experimental autoimmune encephalomyelitis (EAE),[4] a model for multiple sclerosis (MS), can be experimentally induced in several species (guinea pig, mouse, rat, marmoset) by immunization with components derived from the myelin sheath such as myelin basic protein (MBP), proteolipid protein, and myelin oligodendrocyte glycoprotein (MOG) (1). We have previously shown that active immunization of susceptible rat strains with the extracellular part of MOG leads to an ascending paralysis and a histopathology faithfully mimicking the hallmarks of MS in human CNS, i.e., demyelination and axonal loss (2–4). This is in contrast to MBP-induced EAE in rats, which is a monophasic disease without the histopathological hallmarks of MS (1). Moreover, we reported that both MHC genes as well as non-MHC genes have a substantial impact on susceptibility to MOG-induced EAE in rats and on the histopathology, whereby MHC class II effects are relative to and can be overcome by effects of non-MHC genes, other MHC genes like the MHC class I gene products, and environmental influences (2, 5).

MOG is a 218-aa-long glycoprotein exposed on the outer surface of the myelin sheath (6). The physiological function of MOG is still unknown (6). Interestingly, MOG composes only ~0.05% of the myelin sheath and is thought to be CNS specific. Especially the extracellular Ig-like domain of MOG comprising aa 1–125 has gained most interest for neurobiologists and immunologists because of its availability for pathogenic Abs (6, 7). It is encoded within the MHC in humans, mice, and rats (8). There are reports in humans indicating that MOG-directed immunity could cause lesion development in MS (9–15). This is supported by findings in marmosets, rats, and mice (2–4, 16–18). The dissection of genetic and environmental factors leading to detrimental CNS-directed MOG-specific immune attack is a matter of broad interest. Successful manipulations of MOG-directed immunity in a therapeutic sense could potentially benefit MS patients (19). As a prerequisite for successful therapy, target structures for therapy need to be defined that potentially differ in affected individuals depending on genetic allelic variations.

* Experimental Neuroimmunology Laboratory, Department of Neurology, University of Tübingen, Tübingen, Germany; † Neuroimmunology Unit, Center of Molecular Medicine, Karolinska Hospital, Stockholm, Sweden; ‡ Brain Research Institute, Neuroimmunology, University of Vienna, Vienna, Austria; and § Max Planck Institute for Neurobiology, Martinsried, Germany

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[2] Address correspondence and reprint requests to Dr. Robert Weissert, Experimental Neuroimmunology Laboratory, Department of Neurology, University of Tübingen, Hoppe-Seyler-Strasse 3, 72076 Tübingen, Germany. E-mail address: robert.weissert@uni-tuebingen.de

[3] R.W. and K.L.d.G. contributed equally to this work.

[4] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; rMOG, rat MOG; RT1, MHC of rat; MS, multiple sclerosis; LEW, Lewis; ELISPOT, enzyme-linked immunospot; p.i., postimmunization; LN, lymph nodes; MNC, mononuclear cells; CM, complete medium; rrMOG, recombinant rat MOG.

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In this study, we systematically investigated the molecular mechanisms for the MHC haplotype influence on MOG EAE using MHC congenic Lewis (LEW) rats and a set of 18 meric overlapping peptides covering the extracellular part of rat MOG (rMOG). We first focused on the rat MHC class II molecules and assessed how the affinity of the peptide-MHC interaction correlated to disease susceptibility. Subsequently, we mapped the immunodominant and cryptic MOG T cell determinants, assessed linear B cell determinants, and tested for encephalitogenicity of defined determinants in the respective MHC congenic LEW rat strains to elucidate the molecular requirements for induction of EAE.

Although the MHC of rat $(RT1)^l$ and $RT1^u$ haplotypes remained insensitive to MOG-peptide disease induction, in both MOG-EAE-susceptible $RT1^{av1}$ and $RT1^n$ haplotypes disease could equally be induced by MOG-derived peptides. Typically, immunization with these peptides resulted in disease characterized by progressive paralysis and/or ataxia and a histopathology similar to recombinant rat MOG ($rrMOG_{1–125}$) induced EAE. Surprisingly, in the LEW.1N $rMOG_{91–108}$, the disease-inducing peptide, was not the immunodominant epitope as mapped by proliferation or IFN-γ enzyme-linked immunospot (ELISPOT) in peripheral lymphoid tissue. Neither could we demonstrate a T2 biased cytokine response in peripheral lymphoid tissue. Although the absolute need for MOG Abs in demyelination is still controversial (7, 20, 21), we could readily detect an Ab response to $MOG_{91–108}$ in all MHC congenic LEW rat strains immunized with rrMOG, pointing to a role for this stretch as a T and B cell determinant involved in lesion development. Elution of infiltrating cells from the CNS revealed high mRNA expression for IFN-γ, TNF-α, and IL-4 in LEW.1N rats as assessed by quantitative real time PCR, suggesting a localization of peptide-specific reactivated T cells to this compartment.

We demonstrate a powerful MHC class II regulation of MOG autoaggression in which MHC class II peptide binding and peripheral T cell immunodominance fail to predict which autoantigenic peptides may be relevant for an autoaggressive response. Instead, target organ responses may be decisive and should be further explored, as well as the role of overlapping pathogenic T and B cell epitopes and MHC tetramer technology.

Materials and Methods

Rats

Female rats, 10–14 wk of age, were used in all experiments. All strains have been described (2, 22). ACI rats were originally obtained from Harlan Sprague-Dawley (Indianapolis, IN), PVG-$RT1^a$ rats from Harlan U.K. Limited (Blackthorn, U.K.), DA, LEW, LEW.1A, LEW.1AV1, and LEW.1W from the Zentralinstitut für Versuchstierzucht (Hannover, Germany), and LEW.1N, LEW.1AR1, LEW.1AR2, LEW.1WR1, and LEW.1WR2 from H. Hedrich (Medizinische Hochschule, Hannover, Germany). Subsequently, they were locally bred in filter boxes and routinely tested for specific pathogens. Breeding pairs were checked for homozygosity by examination of a microsatellite marker located within the RT1 region.

Synthetic peptides, immunogens, and Ags

The synthetic peptides (Table I) were synthesized by (^{1}H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate strategy (Å. Engström, Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden). Peptides were purified by reversed-phase chromatography and, subsequently, analyzed by plasma desorption mass spectroscopy. The degree of purity of the used peptides was >99%. The reference peptides for the peptide binding assays were N-terminally biotinylated.

rrMOG, corresponding to the N-terminal sequence of rMOG (aa 1–125) was expressed in *Escherichia coli* and purified to homogeneity by chelate chromatography (2, 17). The purified protein in 6 M urea was then dialyzed against PBS to obtain a preparation that was stored at −20°C. rrMOG was used in all cell cultures at a concentration of 3 μg/ml. These Ag concentrations had given optimal stimulations in preceding titration experiments.

Table I. *18 meric 12-aa overlapping rMOG peptides*

Peptide	Sequence
$MOG_{1–18}$	GQFRVIGPGHPIRALVGD
$MOG_{7–24}$	GPGHPIRALVGDEAELPC
$MOG_{13–30}$	RALVGDEAELPCRISPGK
$MOG_{19–36}$	EAELPCRISPGKNATGME
$MOG_{25–42}$	RISPGKNATGMEVGWYRS
$MOG_{31–48}$	NATGMEVGWYRSPFSRVV
$MOG_{37–54}$	VGWYRSPFSRVVHLYRNG
$MOG_{43–60}$	PFSRVVHLYRNGKNQDAE
$MOG_{49–66}$	HLYRNGKNQDAEQAPEYR
$MOG_{55–72}$	KNQDAEQAPEYRGRTELL
$MOG_{61–78}$	QAPEYRGRTELLKESIGE
$MOG_{67–84}$	GRTELLKESIGEGKVALR
$MOG_{73–90}$	KESIGEGKVALRIQNVRF
$MOG_{79–96}$	GKVALRIQNVRFSDEGGY
$MOG_{85–102}$	IQNVRFSDEGGYTCFFRD
$MOG_{91–108}$	SDEGGYTCFFRDHSYQEE
$MOG_{97–114}$	TCFFRDHSYQEEAAVELK
$MOG_{103–120}$	HSYQEEAAVELKVEDPFY
$MOG_{109–126}$	AAVELKVEDPFYWINPGV

Con A was purchased from Sigma (St. Louis, MO) and used in all cell cultures at a concentration of 1 μg/ml.

Induction and evaluation of EAE

The rats were anesthetized by inhalation anesthesia with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL) and injected intradermally at the base of the tail with a total volume of 200 μl inoculum, containing 50 μg rrMOG in saline or, alternatively, 100 μg of peptide emulsified (1:1) with CFA (Sigma) containing 200 μg or, alternatively, 500 μg heat-inactivated *Mycobacterium tuberculosis* (strain H 37 RA; Difco, Detroit, MI).

Rats were scored for clinical signs of EAE and weighed daily up to 40 days postimmunization (p.i.) by two alternating investigators. The signs were scored as follows: grade 1, tail weakness or tail paralysis; grade 2, hind leg paraparesis or hemiparesis; grade 3, hind leg paralysis or hemiparalysis; grade 4, complete paralysis (tetraplegia), moribund state, or death.

Nasal tolerance

After short anesthesia, rats were given in each nostril 60 μg of peptide in 60 μl of PBS on days 11, 10, 9, 8, 7, and 6 before induction of active EAE with rrMOG. Controls received PBS without peptide.

Histopathological evaluation

Histological evaluation was performed on paraformaldehyde-fixed, paraffin-embedded sections of brains and spinal cords (2–4). Paraffin sections were stained with hematoxylin and eosin, Luxol fast blue, and Bielschowsky silver impregnation to assess inflammation, demyelination, and axonal pathology, respectively. An inflammatory index was calculated from the number of perivascular inflammatory infiltrates of each rat on an average of 15 complete cross-sections of spinal cord. The degree of demyelination was evaluated for brain and spinal cord sections separately and semiquantitatively described and scored (2–4). In adjacent serial sections, immunohistochemistry was performed with Abs against the following targets: macrophages/activated microglia (ED1; Serotec, Oxford, U.K.), T cells (W3/13; Seralab, Sussex, U.K.), C9, rat Ig (biotinylated α-rat, Amersham, Little Chalfont, Buckinghamshire, U.K.), and glial fibrillary acidic protein (GFAP; Boehringer- Mannheim, Mannheim, Germany). Bound primary Ab was detected with a biotin-avidin technique. Control sections were incubated in the absence of primary Ab or with nonimmune rabbit serum. The procedures were described (2–4).

Fractionation and cultivation of mononuclear cells (MNC) from lymph nodes (LN) and spleen

Draining inguinal LN were dissected out under deep anesthesia. LN were disrupted and MNC washed twice in DMEM (Life Technologies, Paisley, U.K.), resuspended in complete medium (CM) containing DMEM supplemented with 1% rat serum, 1% penicillin/streptomycin (Life Technologies), 1% glutamine (Life Technologies), and 50 μM 2-ME (Life Technologies) and flushed through a 70-μm plastic strainer (Falcon; BD Biosciences, Franklin Lakes, NJ). MNC from spleen were prepared in the

same way as from LN with the difference that RBC were lysed with lysis buffer consisting of 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2 EDTA adjusted to pH 7.4.

MNC were cultured at a concentration of 2×10^6 cells/ml in either 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark) with 100 μl of cell suspension per well or 24-well flat-bottom plates (Falcon; BD Biosciences) with 1000 μl of cell suspension per well at 37°C in a humidified atmosphere containing 5% CO_2.

Assays of Ag-induced proliferation

All proliferative experiments were performed in triplicate in 96-well round-bottom microtiter plates. MNC (2×10^5/well) in 100 μl CM were cultured with or without the relevant Ag for 60 h and subsequently pulsed with 0.5 mCi [^{3}H]TdR (Amersham Pharmacia Biotech, Uppsala, Sweden) per well for 12 h. DNA was collected on glass fiber filters (Skatron, Sterling, VA), and [^{3}H]TdR incorporation was measured in a beta counter (Beckman Coulter, Fullerton, CA).

Enumeration of cells secreting Ag-specific IFN-γ

To enumerate T cells secreting IFN-γ after Ag exposure, an ELISPOT method was used (2, 23). Nitrocellulose-bottomed 96-well plates (MAHA; Millipore, Molsheim, France) were coated with the mouse mAb DB1 (a generous gift of Peter van der Meide, TNO Primate Center, Rijswijk, The Netherlands), which reacts with rat IFN-γ. Following washing with PBS, the plates were blocked with DMEM containing 5% FCS (Life Technologies). MNC (4×10^5 per well) in 200 μl CM were added to the plates and incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. For each Ag, triplicate determinations were performed. Cells were then discarded, and plates were washed four times with PBS. Secreted and bound IFN-γ was visualized with biotinylated DB12 (also a generous gift of Peter van der Meide), avidin-biotin peroxidase (Vector Laboratories, Burlingame, CA), and subsequently by staining with carbazole (Sigma).

Extraction of CNS cells

Rats were perfused with cold PBS, and brains and spinal cords were dissected out at day 12 p.i. Subsequently, brains and spinal cords were homogenized in 10 ml 50% Percoll/0.1% BSA/1% glucose (Amersham Pharmacia Biotech) containing 500 U DNase type I (Life Technologies) using a B pistil and holder. Ten milliliters of 50% Percoll were added to each sample after homogenization. A discontinuous Percoll gradient was obtained by adding 7 ml of 63% Percoll below and 20 ml of 30% Percoll above the sample. Samples were centrifuged for 40 min at $1000 \times g$ at 4°C. Lymphocytes were collected from the 63/50% Percoll interface. The cells were subsequently washed twice in 15–25 ml PBS with centrifugation at $600 \times g$ for 15 min at 4°C. One fraction of the cells was immediately used for RNA extraction and the other fraction was cultured for 6 h in DMEM/5% FCS/penicillin/streptomycin/glutamine in the presence of Ag.

Determination of anti-MOG serum Abs

Blood samples for Ab measurements were taken at days 12 and 40 p.i. ELISA plates (96-well; Nunc) were coated with 2.5 μg/ml (100 μl/well) rrMOG or 10 μg/ml of peptide (100 μl/well) overnight at 4°C. Plates were washed with PBS/0,05% Tween 20 and blocked for 1 h at room temperature. After washing, diluted serum samples were added and plates were incubated for 1 h at room temperature. Then, plates were washed and rabbit anti-rat antiserum (Nordic, Tilburg, The Netherlands) was added and incubated for 1 h at room temperature. Unbound Abs were removed by washing before the addition of peroxidase-conjugated goat anti-rabbit antiserum (Nordic) diluted in PBS/0.05% Tween 20 (1/10,000). After a 30-min incubation, plates were washed and bound Abs were detected by addition of 3,3′,5′5′-tetramethylbenzidine (TMB, Sigma). The enzymatic reaction was stopped with 1 M HCl after a 15-min incubation in the darkness, and the optical density was read at 450 nm.

ELISA to assess cytokine production in vitro

ELISA kits for detection of IL-4 and IL-10 (BioSource International, Camarillo, CA) were used with supernatants from MNC that had been incubated at a concentration of 2×10^6 cells/ml with or without the relevant Ag or Con A according to the recommended procedures.

cDNA synthesis and quantification of cytokine mRNA levels using real-time PCR

Total RNA was extracted from LN and CNS cells using a RNeasy Mini Kit (Qiagen, Hilden, Germany). To avoid amplification/detection of contaminating genomic DNA, extracted RNA was treated with RNase-free DNase (Promega, Madison, WI). Subsequently, cDNA was synthesized by reverse transcription with Moloney murine leukemia virus reverse transcriptase and random pdN6 primers in the presence of RNase inhibitor (Promega). Amplification was performed on an Applied Biosystems Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR-green method with a two-step PCR protocol (95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). All primers were constructed over exon/exon borders (Table II). Relative quantity of mRNA levels was performed using the standard curve method. The amount of mRNA in each sample was calculated as the ratio between the amount of cytokine and the amount of GAPDH in this sample. For Ag-restimulated cells, the cytokine/GAPDH ratio of the control sample (without Ag) was set to 1 and the ratio of other samples was expressed relative to the control.

Purification of RT1.B and RT1.D molecules

RT1.B and RT1.D molecules were purified from MHC congenic LEW rat (LEW, LEW.1A, LEW.1N, LEW.1W) thymic and splenic tissues by affinity chromatography using OX-6 (specific for RT1.B molecules) and OX-17 (specific for RT1.D molecules) Abs as described (24). Briefly, tissues were lysed in PBS containing 1% Nonidet P-40 (Boehringer Mannheim, Mannheim, Germany) in the presence of protease inhibitors. The lysates were cleared of nuclei and debris by centrifugation at $40{,}000 \times g$ for 60 min and passage over a 45-μm filter. The cleared lysates were cycled over OX-6- and OX-17-coupled cyanogen bromide-activated Sepharose-4B (Pharmacia) columns. The columns were washed with 20 column volumes of PBS/0.1% SDS/0.5% Nonidet P-40, 3 column volumes of PBS/0.05% Nonidet P-40, and 3 column volumes of PBS/1% octyl β-D-glucopyranoside (Sigma). Bound MHC molecules were eluted with 0.05 M diethylamine pH 11/0.15 M NaCl/0.1% octyl β-D-glucopyranoside. After neutralization with 2 M Tris-HCl pH 6.3, the purity of the eluted proteins was assessed by SDS-PAGE and subsequent silver staining. The presence of stable MHC class II complexes for each of the haplotypes was confirmed by running the proteins in SDS-PAGE without denaturation through boiling. The protein content was measured with the BCA protein assay (Pierce, Rockford, IL) using BSA as a standard.

Peptide binding assay

Relative affinities of MOG peptides for purified RT1.B and RT1.D molecules were measured by an inhibition ELISA based on a dissociation-enhanced lanthanide fluoroimmunoassay (Wallac, Turku, Finland) (24). Initially, biotinylated tracer peptides were used in a direct binding assay to establish optimal binding conditions for each of the purified RT1.B and RT1.D molecules. In the inhibition ELISA, RT1.B and RT1.D (50–100 nM) molecules were incubated with fixed amounts of their respective tracer peptides (10–50 nM) used in our preceding study (24) in the presence of a range of dilutions of the unlabeled MOG peptides (10-fold dilutions between 1 nM and 100 μM). pH 5 was the optimal pH for binding of the tracer to most of the purified MHC molecules. The binding buffer consisted of a carbonate buffer titrated to pH 5 containing 2 mM EDTA, 0.01% azide, 0.1 mM PMSF, and 0.1% Nonidet P-40 (Boehringer Mannheim). After an incubation of 48 h at 37°C, the peptide-MHC complexes were transferred

Table II. *Primers for quantitative real-time PCR*

Target Sequence	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
IFN-γ	CCAAGTTCGAGGTGAACAAC	CTCCTTTTCCGCTTCCTTAG
TNF-α	AAATGGGCTCCCTCTCATC	TCCTCTGCTTGGTGGTTTG
IL-4	TGCACCGAGATGTTTGTACC	GAGAACCCCAGACTTGTTCTTC
GAPDH	GGTTGTCTCCTGTGACTTCAAC	CATACCAGGAAATGAGCTTCAC

to Ab-coated (OX-6 or OX-17) ELISA plates (FluoroNunc; Nunc) to remove the excess of nonbound peptides. Europium-labeled streptavidin (Wallac) was added to the plates and incubated for 1 h at room temperature. Finally, the plates were treated with an enhancement solution (Wallac), which releases chelated europium from streptavidin and forms a highly fluorescent solution that can be measured by using a dissociation-enhanced lanthanide fluoroimmunoassay fluorometer (Wallac). The IC_{50} was determined by plotting the percentage of inhibition vs the concentration of added MOG peptide. Peptides were tested in two to three independent experiments.

Results

Binding patterns of overlapping 18 meric rMOG peptides to affinity-purified RT1.B and RT1.D alleles

To investigate how the affinity between peptide and the restricting MHC molecule might contribute to the immunogenic and encephalitogenic potential of certain peptides in the LEW congenic rat strains, we measured relative affinities of the 18 meric rMOG peptides spanning the extracellular part of rMOG for purified rat RT1.B and RT1.D molecules. RT1.B is supposed to be the rat homolog for HLA-DQ or I-A and RT1.D for HLA-DR or I-E. Both the $RT1.B^l$ and $RT1.B^u$ molecules, which are associated with the MOG-resistant haplotype, mainly bound peptides derived from the C-terminal part of rMOG. In contrast, the $RT1.B^a$ and the $RT1.B^n$ molecules displayed affinity for rMOG peptides covering the whole extracellular part of rMOG (Fig. 1). In general, RT1.D molecules bound a broader range of rMOG peptides and with higher relative affinities than the RT1.B molecules (Fig. 1). Although all the alleles studied were capable of binding several rMOG-derived peptides, the $RT1.B^n$ and $RT1.D^n$ molecules were extremely promiscuous binders with almost all of the rMOG peptides binding to both of the molecules.

Mapping of rMOG T and B cell determinants in LEW RT1 congenic rat strains

Next we studied the MHC haplotype-dependent selection of MOG-immunogenic T cell determinants upon immunization with the extracellular rrMOG. Proliferative responses were measured with [^{3}H]TdR uptake and numbers of cells producing IFN-γ by ELISPOT in response to overlapping rMOG sequence-derived peptides (Fig. 2*A*). Peptide-specific responses were in general more sensitively detected by the ELISPOT assay for IFN-γ than proliferation assay. The particular determinants found dominant in the different haplotypes were reproduced in at least four independent experiments. There were different T cell determinants in the four investigated strains, demonstrating the regulation of MHC-related gene products on determinant selection. LEW rats displayed T cell responses to the overlapping peptides MOG_{37-54} and MOG_{43-60}, indicating a dominant determinant in the MOG_{37-60} region. LEW.1AV1 rats displayed T cell responses to the MOG_{73-90} peptide and to the overlapping MOG_{91-108} and MOG_{97-114} peptides. In LEW.1N rats the response to MOG peptide 19–36 was dominant. In contrast, LEW.1W rats did not mount clear-cut responses to any of the peptides.

The strikingly different responses in the $RT1^{av1}$ or $RT1^a$ and $RT1^u$ rats allowed mapping of the T cell responses within the MHC using $RT1^a/RT1^u$ intra-MHC recombinant rats. The $RT1^a$- and $RT1^{av1}$-dependent T cell determinants were purely dependent

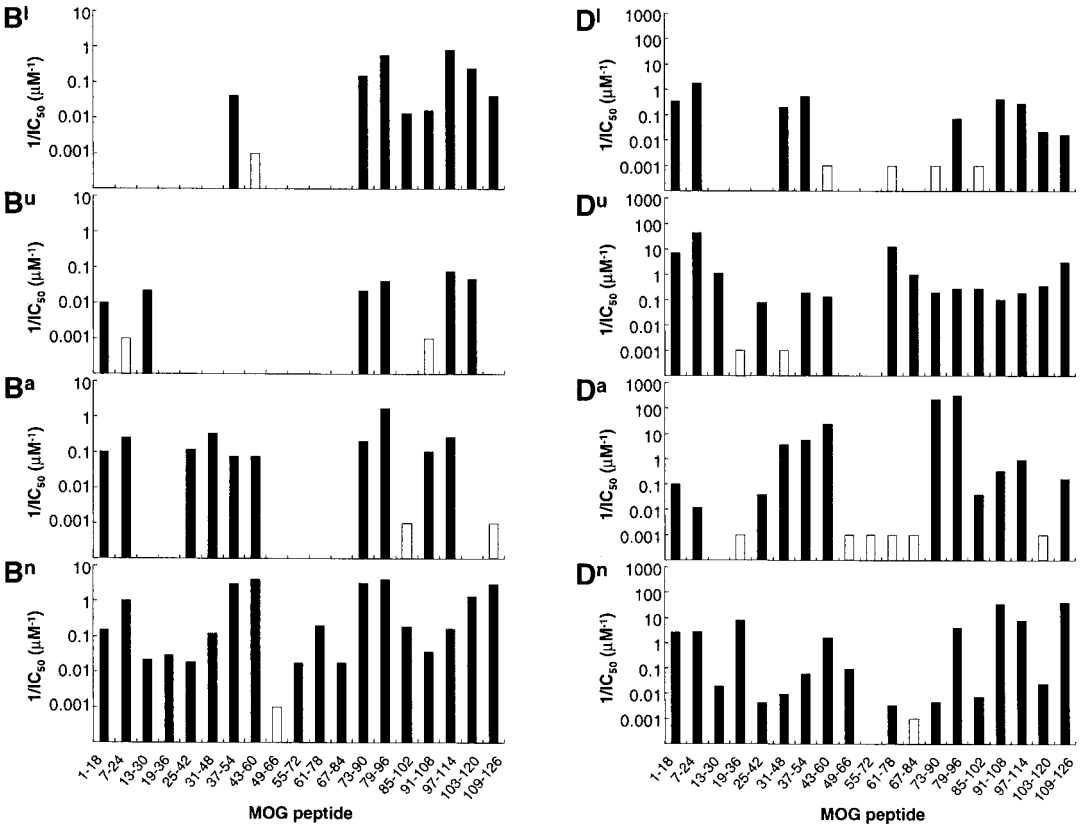

FIGURE 1. Promiscuity of peptide binding to MHC class II molecules of different RT1 haplotypes. Binding of 18 meric 12-aa overlapping rMOG peptides to purified RT1.B (DQ-like) and RT1.D (DR-like) molecules of four different haplotypes was analyzed by ELISA using Europium fluorescence as detection system. IC_{50} values for each of the peptides were derived from the inhibition curves obtained by ELISA as described in *Materials and Methods*. Strong differences in the degree of promiscuity of binding peptides were apparent for the different allelic variants of RT1.B and RT1.D molecules. The measurements for each allele were repeated at least twice.

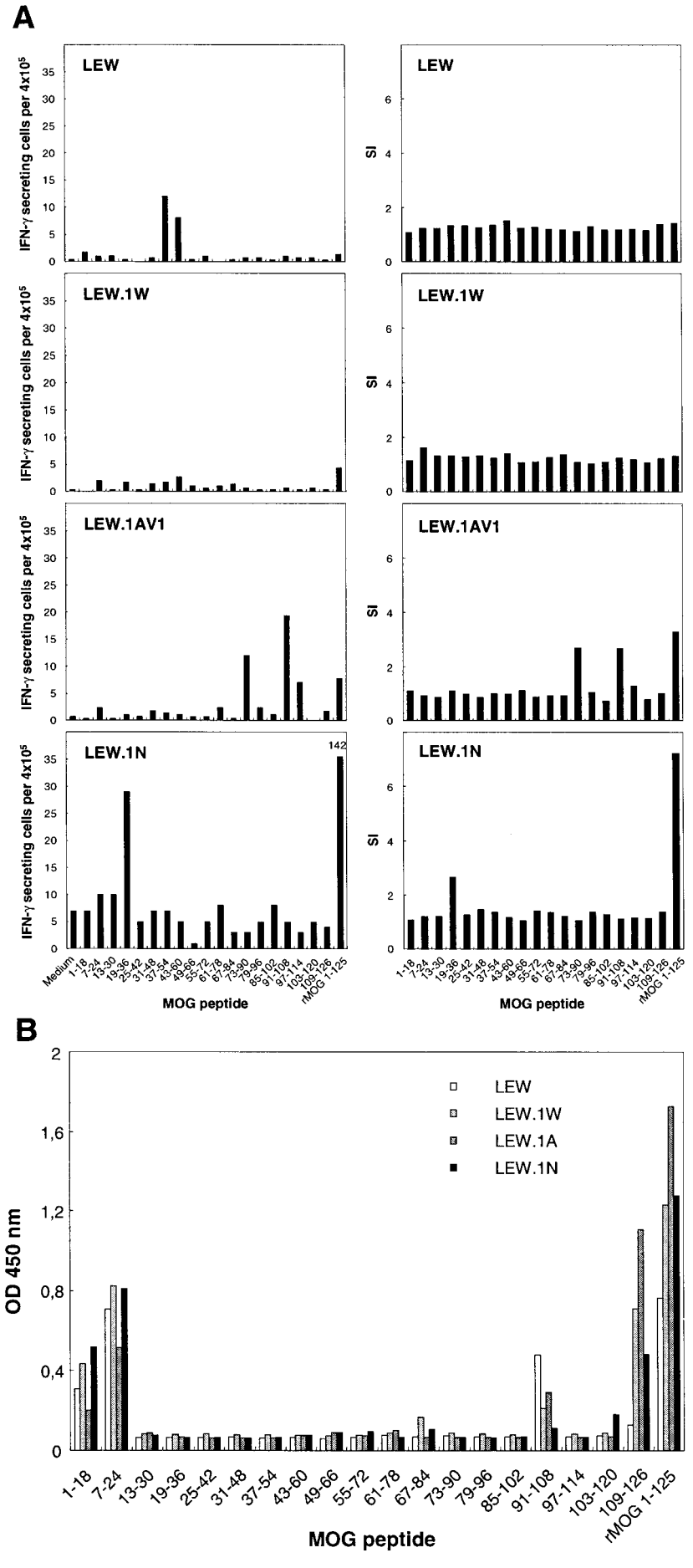

FIGURE 2. T and B cell determinant mapping after active immunization with rrMOG. *A*, T cell reactivities mapped with 18 meric 12-aa overlapping rMOG-derived peptides in short-term cultures on day 12 p.i. with rrMOG immunization. In general, the IFN-γ ELISPOT assay was more sensitive than proliferation. *B*, B cell reactivities to linear rMOG determinants mapped with 18 meric 12-aa overlapping rMOG-derived peptides. Serum samples were taken on day 12 p.i. The bars represent mean values of at least four rats per strain. The procedures were performed as described in *Materials and Methods*.

on the MHC class II region (RT1.B/D) and not on other genes within the MHC (Table III). In addition, non-MHC genes did not affect the MHC-selected immunodominant responses because those were similar in four rat strains with the $RT1^{av1}$ haplotype but different non-MHC genes. There were differences on the qualitative level of proliferative responses and IFN-γ secretion dependent on the non-MHC genes with higher IFN-γ secretion in susceptible strains (data not shown).

We measured Abs of rrMOG-immunized rats to the overlapping MOG peptides to study any MHC haplotype influences on B cell epitope selection. Interestingly, there were reactivities in all strains to peptides MOG_{1-18} and MOG_{7-24}, MOG_{91-108}, and $MOG_{109-126}$ without major MHC-guided predominance (Fig. 2*B*).

MOG peptide-induced EAE

Two sets of MOG peptide immunization experiments, jointly shown in Table IV, were performed. The first set aimed at studying whether the defined dominant determinants were encephalitogenic as well. The second set studied whether cryptic MOG epitopes could be encephalitogenic. In the first set of experiments we immunized groups of LEW rats with peptide MOG_{37-54}, LEW.1AV1 rats with peptides MOG_{73-90} or MOG_{91-108}, and LEW.1N rats with peptide MOG_{19-36}. As shown in Table IV, only LEW.1AV1 rats immunized with MOG_{91-108} induced severe disease and demyelinating lesions. Remarkably, this peptide also proved to be a linear B cell determinant (Fig. 2*B*).

The second set of MOG peptide immunizations studied MHC haplotype regulation of peptide encephalitogenicity systematically, to investigate cryptic determinants as well. Resistance to rrMOG-induced disease in LEW and LEW.1W rats (2) could be due to lack of generation of a suitable encephalitogenic epitope upon Ag processing. To overcome this restriction, we used each of the 19 available rMOG peptides to immunize the different LEW MHC congenic rats (Table IV). Some of the rats were observed until 40 days p.i., whereas others were sacrificed for histopathological examination already on day 16 p.i. No or only mild disease ensued in LEW or LEW.1W rats upon immunization with the peptides. Only one of three LEW rats developed mild disease to peptide MOG_{43-60} and one of eight rats showed a mild inflammation after immunization with the MOG_{37-54}. One of eight rats had mild inflammation in the spinal cord after immunization with the MOG_{91-108} without clinical disease. One of three LEW.1W rats showed slight inflammation of the spinal cord after immunization with peptide MOG_{61-78} without paresis. In contrast, the two MHC congenic strains susceptible to rrMOG, LEW.1AV1 and LEW.1N, displayed conspicuous EAE after immunization with certain peptides. However, these were not congruent with those being immunodominant in regard to T cell immunogenicity. Thus LEW.1AV1 and to our surprise LEW.1N rats showed severe clinical as well as histopathological signs of EAE mainly upon immunization with peptides MOG_{91-108} and MOG_{97-114}, but some milder disease and lesions also with peptides MOG_{79-96} and $MOG_{109-126}$. Furthermore, one of three LEW.1N rats had mild clinical EAE with MOG_{31-48} and one of three rats with MOG_{43-60}, both without detectable infiltrates in the CNS. The clinical signs of EAE induced in diseased rats were rather atypical and included weight loss, severe balance disturbance, and front leg paralysis instead of the more classical flaccid tail and hind leg paralysis described in MBP-induced EAE (2, 3, 23). Disease typically developed between days 15 and 20 p. i., and usually animals recovered and then relapsed in 40% of investigated rats (data not shown). The relapse rate could be higher, but is problematic to assess due to the difficulties in scoring the lesion-associated milder clinical symptoms like optic neuritis, sensory disturbances, and mild bladder dysfunction. The CNS lesions were found with higher incidence in the brain than in the spinal cord. There was a high incidence of optic neuritis. Demyelinating lesions contained complement deposition, indicating Ab-triggered demyelination (Fig. 3). Topography as well as histology mimicked typical MS.

T and B cell responses after MOG peptide immunization in peripheral lymphoid tissue and CNS

To study the immunogenicity of overlapping MOG peptides and to investigate whether this had any relation to encephalitogenicity we investigated quality of peptide-specific T cell response in terms of proliferation as assessed by ^{3}[H]TdR uptake and IFN-γ secretion as assessed by ELISPOT (Fig. 4*A*). The different strains displayed high responses to the immunodominant peptides, defined after MOG immunization. However, in addition there were several

Table III. *Influence of gene products within the MHC on generation of immunodominant T cell determinants in different rat strains after priming with* $rMOG_{1-125}$

RT1	RT1.A	RT1.B/D	RT1.C	Strain	Immunodominant T Cell Determinants
I	I	I	I	LEW	MOG_{37-54}, MOG_{43-60}
u	u	u	u	LEW.1W	None
r4	u	u	a	LEW.1WR1	None
r2	a	u	u	LEW.1AR1	None
a	a	a	a	LEW.1A	MOG_{73-90}, MOG_{91-108}, MOG_{97-114}
r3	a	a	u	LEW.1AR2	MOG_{73-90}, MOG_{91-108}, MOG_{97-114}
r6	u	a	a	LEW.1WR2	MOG_{73-90}, MOG_{91-108}, MOG_{97-114}
av1	a	a	av1	LEW.1AV1	MOG_{73-90}, MOG_{91-108}, MOG_{97-114}
av1	a	a	av1	DA	MOG_{73-90}, MOG_{91-108}, MOG_{97-114}
av1	a	a	av1	COP	MOG_{73-90}, MOG_{91-108}, MOG_{97-114}
av1	a	a	av1	PVG-RTI[a]	MOG_{73-90}, MOG_{91-108}, MOG_{97-114}
av1	a	a	av1	ACI	MOG_{73-90}, MOG_{91-108}, MOG_{97-114}
n	n	n	n	LEW.1N	MOG_{19-36}
n	n	n	n	BN	MOG_{19-36}

[a] The T cell determinant specificity after active immunization with $rrMOG_{1-125}$ is shown in different RT1 haplotypes. The generation of naturally presented dominant T cell determinants was purely dependent on RT1.B/D allelic products and not influenced by other gene products within or outside the MHC. The immunizations and epitope mapping studies were performed as described in *Materials and Methods.*

Table IV. *CNS lesions and disease incidence in four different LEW congenic rat strains actively immunized with overlapping rMOG-derived peptides*[a]

MOG Peptide	LEW				LEW.1W				LEW.1AV1				LEW.1N			
	Disease		Histopathology		Disease		Histopathology		Disease		Histopathology		Disease		Histopathology	
	No.	MS	I	D	No.	MS	I	D	No.	MS	I	D	No.	MS	I	D
1–18	0/3		0,0, 0.1	n	0/3		0/3	n	0/4		0/4	n	0/3		0/3	n
7–24	0/3		0/3	n	0/5		0/5	n	0/4		0/4	n	0/3		0/3	n
13–30	0/3		0/3	n	0/3		0/3	n	0/4		0/4	n	0/3		0/3	n
19–36	0/3		0/3	n	0/2		0/2	n	0/4		0/4	n	0/7		0/7	n
25–42	0/3		0/3	n	0/3		0/3	n	0/4		0/4	n	0/3		0/3	n
31–48	0/3		0/3	n	0/3		0/3	n	0/4		0/4	n	1/3	0,0,2	0/3	n
37–54	0/8		0,0,0,0, 0,0,0 0.06	n	0/2		0/2	n	0/4		0/4	n	0/3		0/3	n
43–60	1/3	0,0,2	0,0, 0.06	n	0/3		0/3	n	0/4		0/4	n	1/3	0,0,1	0/3	n
49–66	0/3		0/3	n	0/3		0/3	n	0/4		0/4	n	0/3		0/3	n
55–72	0/3		0/3	n	0/3		0/3	n	0/4		0/4	n	0/3		0/3	n
61–78	0/3		0/3	n	0/3		0,0, 0.1	n	0/4		0/4	n	0/3		0/3	n
67–84	0/3		0/3	n	0/3		0/3	n	0/4		0/4	n	0/3		0/3	n
73–90	0/3		0/3	n	0/3		0/3	n	0/8		0/8	n	0/3		0/3	n
79–96	0/3		0/3	n	0/3		0/3	n	2/4	0,0,1,1	0, 0.3, 1, 2.4	y	1/3	0,0,2	0/3	n
85–102	0/3		0/3	n	0/3		0/3	n	0/4	0 /4	0/4	n	0/3		0/3	n
91–108	0/8		0,0,0,0, 0,0,0, 0.06	n	0/6		0/6	n	18/19	2.6 ± 0.7	0.5 ± 0.4	y	15/16	2.2 ± 1	1 ± 2.4	y
97–114	0/3		0/3	n	0/3		0/3	n	8/8	2.75 ± 0.7	0.3 ± 0.6	y	2/4	0,0,2,2	0,0, 0.06, 0.06	n
103–120	0/3		0/3	n	0/3		0/3	n	0/4		0/4	n	0/4		0/4	n
109–126	0/3		0/3	n	0/3		0/3	n	3/4	0,1,2,2	0,0,0, 0.06	n	2/5	0,0,0,2, 3	0,0,0, 0.06, 1	n

[a] No., Number of immunized rats per peptide; MS, maximal disease score obtained (scale 1–4); I, presence of inflammation in the CNS, given as inflammatory index; D, demyelination: y, presence (and n, absence) of demyelinating lesions. The immunizations, scorings, and the histopathological procedures were performed as described in *Materials and Methods*.

cryptic determinants in most of the strains giving high T cell responses in the form of IFN-γ and proliferative indices. Although neither LEW nor LEW.1W rats developed disease upon peptide immunizations, both strains were clearly capable of raising T cell responses to several peptides (Fig. 4*A*), which were partly combined with B cell responses to the peptide of immunization (Fig. 4*B*). In LEW.1AV1 as well as LEW.1N rats there were Ab responses against $MOG_{91–108}$ (Fig. 4*B*), which cross-reacted with $rMOG_{1–125}$ (data not shown), suggesting that pathogenic, potentially demyelinating Abs binding to surface-exposed full-length MOG in vivo could have a role in disease induced with $MOG_{91–108}$. Interestingly, there was no detectable Ag-induced T cell response to MOG peptide 91–108 in the LEW.1N rat strain in terms of IL-4 and IL-10 secretion as assessed by ELISA in peripheral lymphoid tissue (data not shown). Real time quantitative PCR for assessment of Ag-induced mRNA expression of IFN-γ, TNF-α, and IL-4 did not reveal T cell reactivity in LEW.1N rats but showed very high IFN-γ mRNA expression in LEW.1AV1 rats (data not shown). There was no Ag-induced TGF-β mRNA detected in LEW.1AV1 and LEW.1N rats (data not shown). Kinetic investigations on days 7, 9, 12, and 16 p.i. did not show T cell responses in LEW.1N rats (each time point $n = 4$). At the time point of immunization, we titrated peptide $MOG_{91–108}$ (1, 5, 10, 20, 50, and 100 μg $MOG_{91–108}$ in CFA at immunization, $n = 4$ rats each peptide dose) to assess the possibility that the height of the Ag dose could have an impact on MOG peptide 91–108-specific recall responses in peripheral lymphoid tissue in LEW.1N rats. Also, this did not result in detection of IFN-γ-secreting cells in peripheral lymphoid tissue (data not shown). Finally, we eluted infiltrating cells from CNS of LEW.1AV1 and LEW.1N rats, enriched these for lymphoid cells with Percoll gradients, and further analyzed them for IFN-γ, TNF-α, and IL-4 mRNA by quantitative real time PCR (Table V). There was mRNA message for IFN-γ, TNF-α, and IL-4 in LEW.1AV1 and LEW.1N rats. The height of the mRNA message was higher in LEW.1N rats compared with LEW.1AV1 rats. This underscores a difference of MHC class II-regulated T cell responses in peripheral lymphoid tissue compared with the target organ tissue in MOG-EAE. Interestingly, restimulation of eluted cells with Ag showed much higher IFN-γ mRNA expression in LEW.1AV1 compared with LEW.1N rats, indicating that T cells had been restimulated in vivo within the target organ to a higher degree in LEW.1N rats compared with LEW.1AV1 rats (Fig. 5).

Intra-MHC region mapping and non-MHC gene influences on MOG peptide 91–108-induced EAE

After demonstrating that MOG peptide 91–108 immunization induced disease in the rats bearing $RT1^{av1}$ and $RT1^{n}$ haplotypes, we were interested in which regions within the MHC permitted disease and whether the peptide-induced disease was subject to non-MHC gene influences. A variety of inbred, MHC congenic, and intra-MHC congenic rat strains were immunized with $MOG_{91–108}$ (Table VI). All congenic and intra-MHC recombinant LEW rat strains with the $RT1.B^{a}/D^{a}$ alleles in the MHC class II region developed disease and histopathological lesions, while rat strains with the $RT1.B^{u}/D^{u}$ alleles in the MHC class II region remained unaffected. There were no apparent influences from the RT1.A or RT1.C region. Immunization of the rat strains with the susceptible

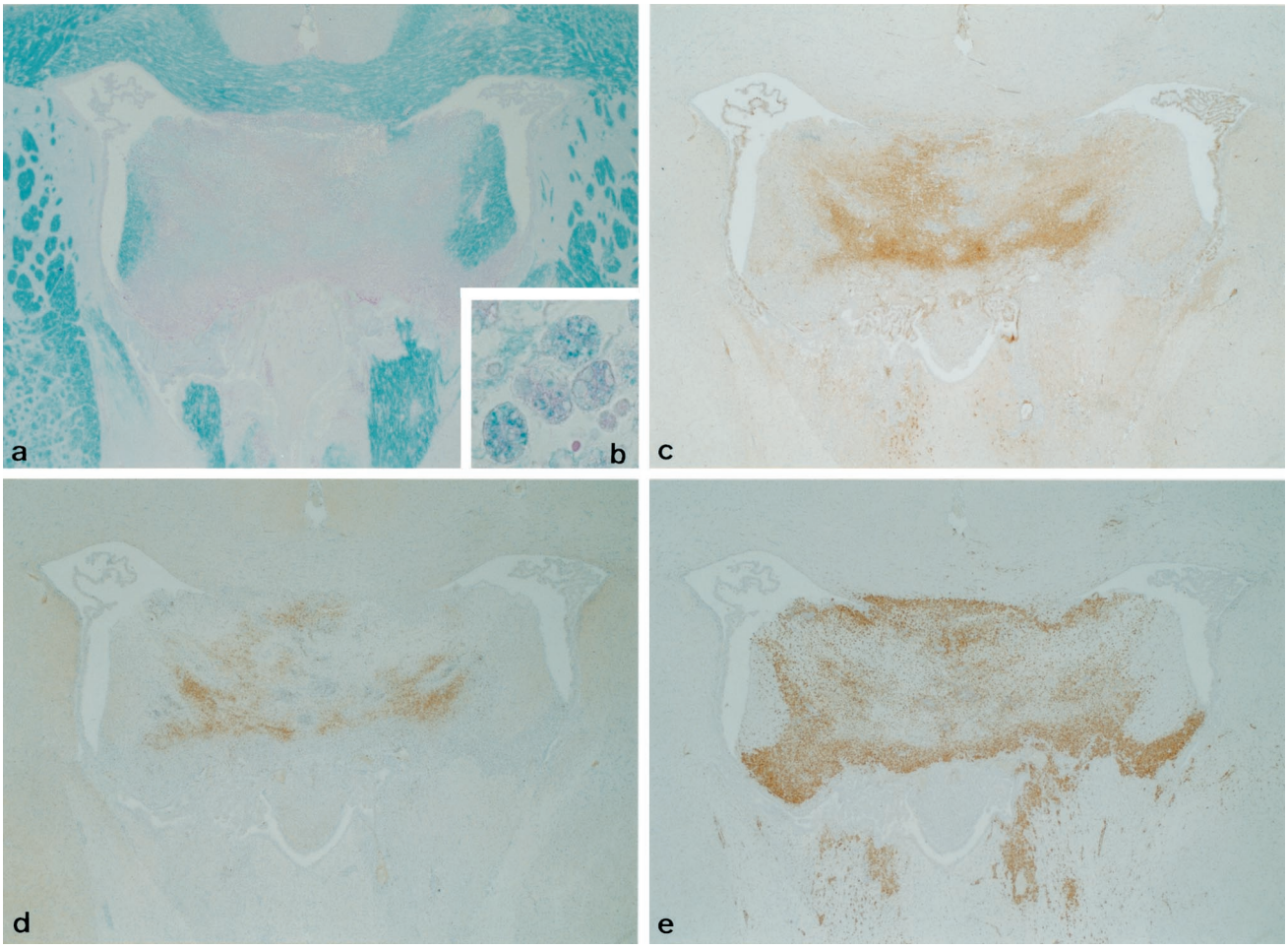

FIGURE 3. Representative histopathology of LEW.1AV1 and LEW.1N rats immunized with MOG peptide 91–108. Large confluent demyelinated lesion located in the fornix (*a*). Higher magnification shows myelin degradation products positive for luxol fast blue in macrophages (*b*). Active demyelination is associated with deposition of complement component C9 (*c*) and T cell and granulocyte infiltration (*d*); inactive areas contain predominantly macrophages (*e*). Serial diencephalic brain sections were stained with luxol fast blue (*a* and *b*); immunohistochemistry for complement component C9 (*c*), for W3/13 (T cells and granulocytes, *d*), and for ED1 (activated macrophages and microglia, *e*). Magnification: *a*, *c*, *d*, and *e*, ×30; *b*, ×75.

RT1.B^a/D^a alleles in the MHC class II region, to study non-MHC gene regulation, demonstrated that all investigated dark Agouti rats developed disease, whereas all five PVG-RT1^a rats were protected and only one of four ACI rats had some mild CNS infiltrates. Thus, non-MHC genes regulate MOG peptide 91–108-induced EAE.

Nasal tolerance induction with either MOG$_{19–36}$ or MOG$_{91–108}$ in LEW.1N rats immunized with rrMOG

To prove that MOG$_{91–108}$ contains the disease-inducing epitope in LEW.1N rats after immunization with rrMOG we nasally tolerized with MOG$_{19–36}$, MOG$_{91–108}$, or (as control) PBS and induced active EAE with rrMOG. Only LEW.1N rats nasally tolerized with MOG$_{91–108}$ showed amelioration of EAE, in contrast to LEW.1N rats tolerized with MOG$_{19–36}$, which developed the same severity of disease as PBS-treated controls (Fig. 6). This experiment strongly suggests that MOG$_{91–108}$ is the disease-promoting MOG determinant in LEW.1N rats immunized with rrMOG$_{1–125}$.

Discussion

Based on this study we conclude that 1) the immune response against encephalitogenic sequences can differ dramatically in peripheral lymphoid tissue compared with the target organ tissue, the CNS (immunodominance in peripheral lymphoid tissue in MOG-EAE is not linked to disease-inducing potential). Immunization with MOG$_{19–36}$ failed to induce disease in LEW.1N, although the ex vivo response to this peptide was dominant in the RT1^n haplotype. Rather, overt clinical disease accompanied by CNS lesions could be induced in this strain with MOG$_{91–108}$, a peptide to which no IFN-γ response or T2-biased cytokine response could be measured in peripheral lymphoid tissue but in the CNS); 2) MOG region 91–114 contains the autoaggressive or disease-promoting T cell determinants in LEW.1AV1 as well as LEW.1N rats; 3) the generation of a peptide-specific IFN-γ response or B cell reactivity was not sufficient to induce disease (for example, LEW rats did not develop disease after immunization with MOG$_{91–108}$, nor did LEW.1W rats develop disease after immunization with MOG$_{1–18}$, although in both cases immunization with these peptides led to an IFN-γ response and an Ab response that was cross-reactive with rrMOG (data not shown)). Possibly, this is due to lack of processing of these peptides in the CNS or to the presence of a regulatory subset of T cells, Ref. 25–27); and 4) although binding of MOG peptides is a prerequisite for immunogenicity, the predictive value of the MHC class II binding for encephalitogenicity is low. This is in line with earlier studies in proteolipid protein-induced EAE in mice (28). In contrast to this study we show also that peptides without a detectable ex vivo T cell response can induce EAE.

Immunization with MOG$_{91–108}$ not only led to different outcomes in the four tested LEW RT1 congenic rat strains. Non-MHC

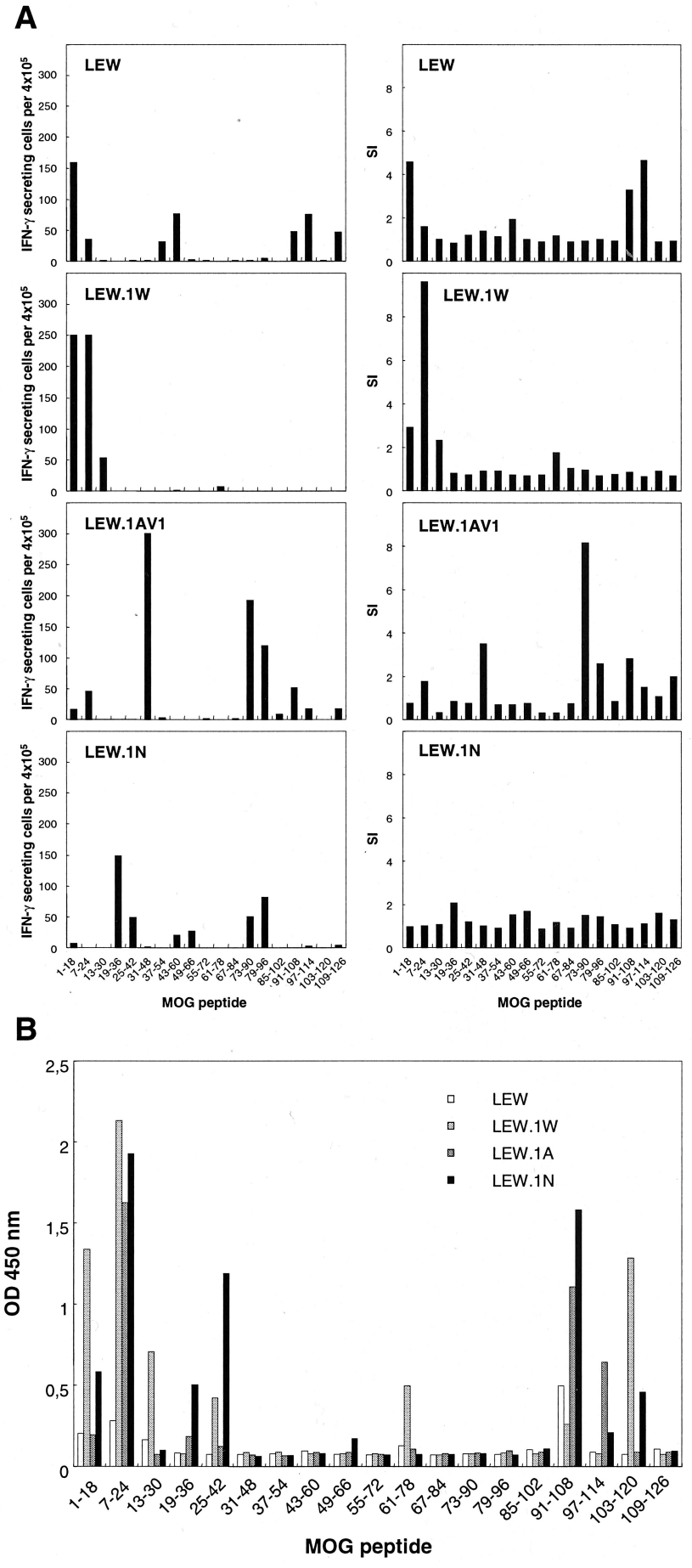

FIGURE 4. T and B cell repertoire analysis to MOG determinants. The figure illustrates actively immunized rats with 18 meric 12-aa overlapping rMOG-derived peptides spanning the extracellular domain of rMOG. *A*, T cell reactivities analyzed on day 16 p.i. by ELISPOT assay for IFN-γ-secreting cells and proliferation to the peptide of immunization. *B*, Serum samples taken and analyzed by ELISA for rMOG peptide-specific Abs taken on day 16 p.i. to the peptide of active immunization. The bars represent mean values of at least four rats per strain. The procedures were performed as described in *Materials and Methods*.

Table V. *Cytokine mRNA expression of CNS-infiltrating cells assessed by quantitative real-time PCR*[a]

Strain	Cytokines		
	IFN-γ/GAPDH	IL-4/GAPDH	TNF-α/GAPDH
LEW.1AV1	1.6 ± 0.14	9.1 ± 1.3	1.7 ± 0.1
LEW.1N	13.5 ± 2.6	87.3 ± 34	17.4 ± 3
p	0.01	0.08	0.006

[a] CNS-infiltrating cells were rescued on day 12 p.i. by Percoll gradients and subsequently analyzed for cytokine mRNA expression by quantitative real time PCR for IFN-γ, TNF-α, and IL-4. There was detectable mRNA expression for IFN-γ, TNF-α, and IL-4. Cells eluted from LEW.1N rats showed quantitatively higher cytokine mRNA expression compared to LEW.1AV rats. The procedures were performed as described in *Materials and Methods. p*, Statistical significance in the comparison LEW.1AV1 to LEW.1N.

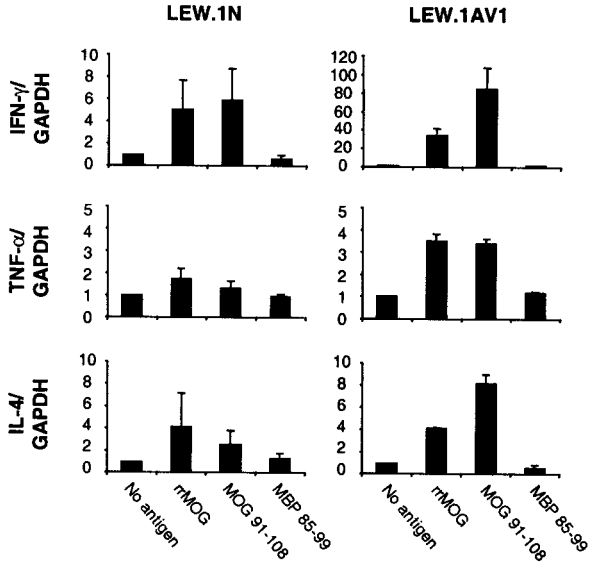

FIGURE 5. Ag-induced cytokine mRNA expression of CNS-infiltrating cells. Ag-induced IFN-γ, TNF-α, and IL-4 mRNA expression of CNS-infiltrating cells is illustrated as assessed by real time quantitative PCR. Rats were sacrificed on day 12 p.i. CNS-infiltrating cells were eluted over Percoll gradients, and subsequently eluted cells were restimulated for 6 h with and without Ag. The bars represent mean values of four rats per strain. The procedures were performed as described in *Materials and Methods*.

genes affected disease and immune responses as well: in four tested $RT1^{av1}$ congenic rat strains, all strains showed responses to this peptide by proliferation, but differences in IFN-γ secretion (data not shown) and disease susceptibility. These differences are the starting point of investigations on both MHC and non-MHC gene-mediated regulation of $MOG_{91–108}$-specific responses and susceptibility (5).

Immunodominance of T cell determinants was purely MHC class II molecule guided as we could show in epitope mapping studies after active immunization with $rrMOG_{1–125}$ of different MHC congenic, intra-MHC congenic, and inbred rats with different non-MHC genes (29). This observation points to the MHC molecule and its physicochemical properties as major determinator and 'nursing' structure for selection of peptides during Ag processing and subsequent presentation to T cells (30). The spectrum of MOG peptides binding to the different allelic variants of RT1.B molecules varied greatly, but to a lower degree for the RT1.D molecules. This is in line with our preceding work in MBP-induced EAE in different MHC congenic LEW rat strains (24). Certain haplotypes, like the $RT1^n$ haplotype, displayed a very promiscuous binding of MOG peptides. All immunogenic peptides bound with intermediate or high affinity to either one or both of the RT1.B or RT1.D molecules. But although several peptides bound well to the MHC molecules, many of these were nonimmunogenic or nonencephalitogenic. It demonstrates that the autoimmune responses to many parts of this protein are tolerized (31–33). Because, to our current knowledge, MOG is sequestered in the CNS, tolerization could be achieved by homologous proteins such as the butyrophylin and B7 family (8, 34) or host mimicking exogenous agents (23, 35, 36).

Our data argue for differential selection and/or tuning of MOG sequence 91–114- specific pathogenic and regulatory T cells in different MHC haplotypes during tolerance induction or peripheral activation (37). The origin of this haplotype-dependent effect might lie in the level of promiscuity of MOG-derived peptides binding to the haplotype-associated MHC class II molecules and the affinities of these interactions. The lack of a measurable T cell response to $MOG_{91–108}$ in LEW.1N rats in peripheral lymphoid tissue but the presence of such a response in the CNS target tissue argues for breakage of tolerance within the target tissue. The exact mechanism that leads to intra-CNS expansion of autoreactive T cells is presently unclear and the subject of ongoing investigations.

Potentially very small numbers of $MOG_{91–108}$ autoreactive T cells could be present in peripheral lymphoid tissue that get activated by challenge with Ag but that are not detected ex vivo. They

Table VI. *MOG peptide 91–108-induced EAE*[a]

RT1	RT1.A	RT1.B/D	RT1.C	Strain	Disease	Lesions
I	I	I	I	LEW	0/8	1/8
u	u	u	u	LEW.1W	0/6	0/6
r4	u	u	a	LEW.1WR1	0/4	0/4
r2	a	u	u	LEW.1AR1	0/7	0/7
a	a	a	a	LEW.1A	7/10	8/10
r3	a	a	u	LEW.1AR2	5/5	5/5
r6	u	a	a	LEW.1WR2	4/4	4/4
av1	a	a	av1	LEW.1AV1	18/19	8/10
av1	a	a	av1	DA	4/5	4/5
av1	a	a	av1	PVG-RTI[a]	0/5	0/5
av1	a	a	av1	ACI	1/4	1/4
n	n	n	n	LEW.1N	15/16	7/9

[a] The incidence of CNS lesions and incidence of disease is shown in different rat strains after active immunization with MOG peptide 91–108. The development of CNS lesions in the intra-MHC recombinant rat strains was purely dependent on RT1.B/D gene products. The immunizations, scoring, and histopathological investigations were performed as indicated in *Materials and Methods*.

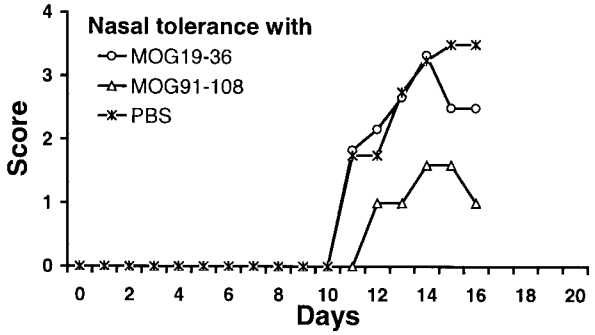

FIGURE 6. Nasal tolerance with either MOG_{19-36} or MOG_{91-108} in rrMOG-induced EAE. Rats were given in each nostril 60 μg of peptide in 60 μl of PBS on days 11, 10, 9, 8, 7, and 6 before induction of active EAE with rrMOG. Only LEW.1N rats that had received MOG_{91-108} ($n = 7$) showed significant reduction in disease score ($p = 0.008$), whereas MOG_{19-36} ($n = 7$)- or PBS ($n = 7$)-treated rats were not protected. The procedures were performed as described in *Materials and Methods*.

travel into the CNS where they get restimulated and expanded by Ag presented on local APCs. APCs in the CNS might have different stimulatory capacity compared to APC in peripheral lymphoid tissue. The MOG_{91-108}-specific T cells could also be partially tolerized and express a cytokine or chemokine that we did not investigate.

A discrepancy lies in the fact that, when mapping dominant determinants, immunization of LEW.1N rats with extracellular MOG systematically leads to large amounts of IFN-γ-secreting cells in peripheral lymphoid tissue, whereas none of the extracellular MOG peptides can equal this response. This is also in contrast with the $RT1^{l}$ and $RT1^{av1}$ haplotypes, where the extracellular MOG response is generally lower than in the $RT1^{n}$ haplotype, but where the dominant determinants show a very clear-cut IFN-γ response (Fig. 2). Because both extracellular MOG and MOG_{91-108} can induce severe EAE in LEW.1N rats, this demonstrates that IFN-γ response and ensuing disease do not always correlate. Because rMOG requires protein processing for the generation of an autoantigen-specific T cell response, the role of disease-associated IFN-γ might lie in the diversification of the immune response in forms of increased processing and presentation of different rMOG determinants through increased engagement of a variety of APC-like B cells (38–40). This might lead to the diversification of the MOG specific immune response by presentation of 'hidden' epitopes (41). Moreover, IFN-γ production of T cells might stimulate the production of autoantibodies to conformationally dependent or linear epitopes of MOG (42).

To obtain a pathogenic/encephalitogenic autoimmune response upon immunization with $rrMOG_{1-125}$, the response needs diversification toward a determinant within MOG sequence 91–114 because this peptide caused severe histopathological lesions and disease in LEW.1AV1 and LEW.1N rats, regardless of being immunodominant or cryptic. A constitutive level of presentation of this T cell determinant in the CNS is required, otherwise EAE would not develop after immunization with peptides MOG_{91-108} and MOG_{97-114} (43). Moreover, MOG_{91-114}-reactive T cells must pass the blood-brain barrier, what preferentially happens if T cells are activated and express the right set of accessory molecules like adhesion molecules (44–46). Our data indicate that in strains that do not show strong T cell reactivity toward these peptides in the peripheral lymphoid tissue, potentially a strong response at the target organ might exist. This response would be dependent on the local APC and their naturally processed and presented peptide determinants (43, 47).

Both susceptible LEW.1AV1 and LEW.1N strains, as well as the resistant LEW and LEW.1W strains, raised a B cell response upon immunization with MOG peptide 91–108, which was cross-reactive with rMOG (data not shown). Moreover, complement deposition was visible in most of the MOG_{91-108}- and MOG_{97-114}-immunized animals, suggesting Ab-triggered demyelination (3, 48, 49). Recently, Genain et al. were able to identify Abs against linear MOG epitopes specifically binding to disintegrating myelin around axons in lesions of acute MS (15). Taken together, these data strongly point toward a pathogenic potential of Abs against MOG_{91-108} and MOG_{97-114}. If these Abs are involved in demyelination (7, 20), this would argue for a qualitative or quantitative difference in the Ab response raised against this peptide dictated by elements within the MHC, which map toward the RT1.B/D region. This might be due to differences in T cell help toward B cells. Alternatively, but not in line with our data, T effector cells might mediate B cell-independent demyelination (21).

In our study, only MOG sequence 91–114 reproducibly induced CNS inflammation and demyelination associated with severe clinical disease. This is in contrast to other studies where also MOG_{35-55} induced demyelination and disease in LEW rats (50). The discrepancy might lie in the use of different substrains of LEW rats. Interestingly, MOG_{35-55} is considered one of the main encephalitogenic regions of MOG because it is capable of inducing MS-like disease in several mouse strains (18, 51). These studies only tested MOG peptides predicted by computer programs that did not identify MOG_{91-108} as a potential encephalitogenic T cell determinant. An exception is the work of Amor et al., who identified MOG_{92-106} as encephalitogen in SJL mice by systematic immunization with overlapping peptides covering the extracellular part of MOG (17).

The data have implications for studies of potential pathogenic autoimmune T cell responses in humans in the sense that in this study neither peptide binding nor epitope mapping with proliferation and IFN-γ secretion, T2 cytokine ELISA, and mRNA expression for cytokines were capable of identifying all encephalitogenic determinants in peripheral lymphoid tissue. Current approaches for detection of disease inducing T cells in the periphery in humans might only reveal a limited and not relevant set of epitopes. The data underscore the need to investigate cellular responses within the target organ tissue.

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Publikation 5

T Cell Epitopes of Human Myelin Oligodendrocyte Glycoprotein Identified in HLA-DR4 (DRB1*0401) Transgenic Mice Are Encephalitogenic and Are Presented by Human B Cells[1]

Thomas G. Forsthuber,[2]* Carey L. Shive,* Wolfgang Wienhold,† Katrien de Graaf,† Edward G. Spack,‡ Robert Sublett,§ Arthur Melms,† Jens Kort,* Michael K. Racke,¶ and Robert Weissert†

Myelin oligodendrocyte glycoprotein (MOG) is an Ag present in the myelin sheath of the CNS thought to be targeted by the autoimmune T cell response in multiple sclerosis (MS). In this study, we have for the first time characterized the T cell epitopes of human MOG restricted by HLA-DR4 (DRB1*0401), an MHC class II allele associated with MS in a subpopulation of patients. Using MHC binding algorithms, we have predicted MOG peptide binding to HLA-DR4 (DRB1*0401) and subsequently defined the in vivo T cell reactivity to overlapping MOG peptides by testing HLA-DR4 (DRB1*0401) transgenic mice immunized with recombinant human (rh)MOG. The data indicated that MOG peptide 97–108 (core 99–107, FFRDHSYQE) was the immunodominant HLA-DR4-restricted T cell epitope in vivo. This peptide has a high in vitro binding affinity for HLA-DR4 (DRB1*0401) and upon immunization induced severe experimental autoimmune encephalomyelitis in the HLA-DR4 transgenic mice. Interestingly, the same peptide was presented by human B cells expressing HLA-DR4 (DRB1*0401), suggesting a role for the identified MOG epitopes in the pathogenesis of human MS. *The Journal of Immunology,* **2001, 167: 7119–7125.**

Multiple sclerosis (MS)[3] is a chronic inflammatory and demyelinating disease of the CNS believed to be mediated by an autoimmune T cell response directed at proteins of the myelin sheath, such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (1–5). Although considerable research has focused on MBP, observations of a predominant MS patient T cell response to MOG, and MS-like lesions in marmosets immunized with MOG have heightened interest in this Ag (6–8). Knowledge of the MOG epitopes targeted by the T cell response in patients would be important for the understanding of the pathogenesis of MS, and to identify possible candidate peptides for immune intervention. However, the analysis of Ag-specific T cell responses in genetically diverse MS patients has remained technically challenging. Frequencies of Ag-specific T cells in freshly isolated human tissues, such as peripheral blood, are frequently at or below the detection limit of current technologies (9). Therefore, the exact nature of the T cell epitopes of MOG presented by MHC molecules in MS patients has remained unresolved. The human MHC molecules most frequently associated with MS are HLA-DR2 (DRB1*1501, DRB5*0101, and DQB1*0602) (10, 11) and HLA-DR4 (DRB1*0401) in a subpopulation of patients with Mediterranean background (10–13).

MHC molecules are highly polymorphic and have unique binding motifs. Therefore, results on determinant usage obtained in conventional animal models cannot necessarily be extrapolated to Ags presented in the context of human MHC molecules (14). To overcome these limitations, we have applied MHC binding algorithms to predict MOG peptide binding to HLA-DR4 (DRB1*0401), and subsequently probed HLA-DR4 (DRB1*0401) transgenic mice immunized with rhMOG for T cell reactivity to overlapping MOG peptides by computer-assisted cytokine ELISPOT assays at single cell resolution. The data showed that MOG peptide (p)97–108 (core 99–107, FFRDHSYQE) was the immunodominant HLA-DRB1*0401-restricted T cell epitope. This region had a high in vitro binding affinity for HLA-DR4, showed complete sequence homology among mice, rats, and humans, and induced severe experimental autoimmune encephalomyelitis (EAE) in the HLA-DR4 transgenic mice. Interestingly, the same peptide was presented by human HLA-DR4 homozygous B cells, suggesting a role for this epitope in the pathogenesis of MS.

Materials and Methods

Mice, Ags, and injections

HLA-DR4 (DRB1*0401) transgenic mice were generated as described previously (15) and bred at Case Western Reserve University (Cleveland, OH)

*Institute of Pathology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106; †Department of Neurology, University of Tübingen, Tübingen, Germany; ‡InterMune, Brisbane, CA 94010; §Custom Computer Software, Foster City, CA 94404; and ¶Department of Neurology, University of Texas-Southwestern Medical Center, Dallas, TX 75235

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[2] Address correspondence and reprint requests to Dr. Thomas G. Forsthuber, Institute of Pathology, School of Medicine, Case Western Reserve University, BRB 936, 2109 Adelbert Road, Cleveland, OH 44106-4943. E-mail address: tgf2@pop.cwru.edu

[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; MBP, myelin basic protein; rh, recombinant human; CLIP, class II-associated invariant chain peptide; p, peptide.

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under special pathogen-free conditions. Female transgenic mice were injected at 6–10 wk of age with the Ag in CFA. Pertussis toxin (List Biological Laboratories, Campbell, CA) was added to the immunization regimen as indicated. Recombinant MOG was prepared as described subsequently. Overlapping MOG peptides were obtained from Princeton Biomolecules (Langhorne, PA). CFA was prepared by mixing IFA (Life Technologies, Grand Island, NY) with *Mycobacterium tuberculosis* H37RA at 5 mg/ml (Difco, Detroit, Michigan). Ags were mixed with the adjuvant to yield a 2-mg/ml emulsion, of which 50 μl was injected s.c. as specified.

Cell separations

Single cell suspensions were prepared from HLA-DR4 lymph node cells. $CD4^+$ or $CD8^+$ T cells were obtained by passing the cells through a murine $CD8^+$ or $CD4^+$ T cell enrichment column (R&D Systems, Minneapolis, MN) following the manufacturer's suggested protocol. FACS analysis showed >95% enrichment for either $CD4^+$ or $CD8^+$ T cells. Irradiated syngeneic spleen cells or EBV-transfected HLA-DR4 (DRB1*0401) homozygous B cells (16) were added at 1×10^5 cells per well as indicated in Fig. 5.

Cytokine measurements by ELISPOT and computer-assisted ELISPOT image analysis

Cytokine ELISPOT assays were performed as described previously (17). Briefly, ELISPOT plates (ImmunoSpot; Cellular Technology, Cleveland, OH) were coated overnight with IFN-γ (R46A2, 4 μg/ml)- or IL-5 (TRFK5, 5 μg/ml)-specific capture Ab diluted in 1× PBS. The plates were blocked with 1% BSA in PBS for 1 h at room temperature, then washed four times with PBS. Cells from draining lymph nodes were plated at 5×10^5 cells/well alone or with MOG peptides (7 μM) in HL-1 medium supplemented with 1% L-glutamine and cultured for 24 h for IFN-γ and 48 h for IL-5. Subsequently, the cells were removed by washing four times with PBS then four times with PBS/Tween, and the biotinylated detection Ab XMG1.2-biotin (2 μg/ml) for IFN-γ or TRFK4-biotin (2 μg/ml) for IL-5 were added and incubated overnight. The plate-bound second Ab was then visualized by adding streptavidin-alkaline phosphatase (DAKO, Carpenteria, CA) and nitroblue tetrazolium (Bio-Rad, Hercules, CA)/5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma-Aldrich, St. Louis, MO). Image analysis of ELISPOT assays was performed on a Series 1 ImmunoSpot Image Analyzer (Cellular Technology) as described previously (17, 18). In brief, digitized images of individual wells of the ELISPOT plates were analyzed for cytokine spots based on the comparison of experimental wells (containing T cells and APC with Ag) and control wells (T cells and APC; no Ag). After separation of spots that touched or partially overlapped, nonspecific noise was gated out by applying spot size and circularity analysis as additional criteria. Spots that fell within the accepted criteria were highlighted and counted. Stimulation index was calculated by dividing the number of cytokine spots detected in wells pulsed with relevant Ag by the number of cytokine spots in wells without Ag (medium only). The spot number in unimmunized or control mice (irrelevant Ag) was in the same range as the medium controls (not shown). Statistical analysis was performed with the paired *t* test or the Mann-Whitney rank sum test using SigmaStat software (SPSS, Chicago, IL).

Evaluation of clinical disease

Mice were monitored daily for 30 days and on alternate days thereafter. A mean clinical score was assigned for each group using the following scale (19): 0, no abnormality; 1, limp tail; 2, moderate hind limb weakness; 3, complete hind limb paralysis; 4, quadriplegia or premoribund state; 5, death.

Histopathology

At the time of the experiment the brain and spinal cord of the mice were removed and either preserved in 10% formalin or snap-frozen in 2-methylbutane. Five-micrometer slices of the CNS tissue were prepared and stained with H&E. The tissue was then examined by light microscopy in a blinded fashion by a neuropathologist and evaluated for the extent of inflammation and graded as follows: −, no inflammation; +/−, a few mononuclear cells; +, organization of inflammatory infiltrates around positive vessels; ++, extensive perivascular cuffing with extension into the subarachnoid space; and +++, extensive perivascular cuffing with increasing subarachnoid inflammation (19, 20). Immunofluorescence staining of the brain tissue was performed as described (21). In brief, 5-μm sections of snap-frozen brain tissue were fixed with 4% paraformaldehyde and probed with FITC- or PE-conjugated anti-mouse CD3, CD4, CD8, CD19, MAC-1, and F4/80 Abs (BD PharMingen, San Diego, CA). Images of Ab-labeled tissue sections were captured using a Leica fluorescence microscope equipped with a charge-coupled device camera and image analysis software. Sections were analyzed in a blinded fashion.

*Prediction of MOG sequences with high affinity for HLA-DRB1*0401*

To identify potential core peptides with predicted high binding affinity for HLA-DRB1*0401, the MOG amino acid sequence was analyzed with two published HLA-DR4 binding algorithms (22, 23). Briefly, a computer program was written that parsed the MOG sequence into successive 9-mers, each beginning one amino acid after the start of the previous 9-mer. The contribution toward binding of each amino acid was summed based on the matrix published by Hammer et al. (22), yielding a peptide score. Higher peptide scores indicate relatively higher affinity binding to HLA-DRB1*0401. Several immunodominant peptides documented in the literature yield relative affinity scores in the range of 4–6 (22). This hierarchy of predicted high affinity peptides was compared with predictions based on the published algorithm of Marshall et al. (23), which calculates an IC_{50} concentration rather than a relative score. The Marshall et al. (23) algorithm was based on the effects of single amino acid substitutions within the 11-residue core of a 13-mer polyalanine peptide (AAYAAAKAAAAAA) that binds promiscuously to most HLA-DR alleles. The contributions of each of the 11 amino acid residues were multiplied consecutively, and the sum was multiplied by the IC_{50} of the parent polyalanine peptide (14.7 nM) to derive a predicted IC_{50} for the sequence. Lower IC_{50} values indicate higher predicted affinity of a peptide containing the sequence. To compare the affinity of an amino acid sequence predicted by the two algorithms, 9-mer sequences with the anchor residue (F, I, L, M, V, W, or Y) at position 1 were first evaluated by the Hammer et al. (22) algorithm. Each 9-mer, along with the N- and C-flanking amino acids, were then evaluated by the Marshall et al. (23) algorithm, providing an 11-mer peptide with the anchor residue (F, I, L, M, V, W, or Y) at the second position as required by this algorithm.

In vitro peptide binding studies

In vitro binding competition assays were performed as described previously (24). In brief, biotinylated tracer peptides were used in a direct binding assay to establish optimal binding conditions for the purified HLA-DR4 (DRB1*0401) molecules. Relative affinities of MOG peptides for the HLA-DR4 molecules were assessed by an inhibition ELISA based on a dissociation-enhanced lanthanide fluoro-immunoassay (DELFIA; Wallac, Turku, Finland). In the inhibition assay, HLA-DR4 molecules (10 nM) were incubated with fixed amounts of the tracer peptide (class II-associated invariant chain peptide (CLIP)97–120) in the presence of various concentrations (10-fold dilutions between 1 nM and 100 μM) of the unlabeled MOG peptides. The concentration yielding 50% inhibition of binding of the tracer peptide (IC_{50}) was measured by plotting the percentage of inhibition vs the concentration of MOG peptide. Peptides were tested in three independent experiments.

Cloning and bacterial expression of human MOG

The cDNA encoding amino acids 23–247 of human MOG was obtained by reverse transcription of total RNA from the human glioma cell line 45/8. RNA was prepared with TRIzol Reagent (Life Technologies) and first-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase, RNase H Minus (Promega, Madison, Wisconsin), and the gene-specific primer R785. The amplified PCR product was separated by agarose gel electrophoresis on a 1.2% gel and purified with a QIAEXII kit (Qiagen, Hilden, Germany). The cDNA fragment was cloned in Bluescript II KS^+ vector (Stratagene, La Jolla, California), which was previously treated with *Sma*I and alkaline phosphatase according to conventional protocols. The nucleotide sequences of the various clones were determined for both strands by using the PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit in conjunction with an Applied Biosystems model 310 DNA Sequencer (both from Applied Biosystems, Foster City, CA). After the purification of the resulting PCR product by phenol:chloroform extraction and ethanol precipitation, the fragment was digested with *Nco*I and *Bam*HI and ligated into the *Nco*I/*Bam*HI linearized pQE60 vector (Qiagen). To improve the bacterial expression level, it was necessary to replace some of the original, N-terminal MOG codons by PCR mutation. The nucleotide sequences of the resulting clones were confirmed by sequence analysis, and clone mog-1His was used for expression in *Escherichia coli*. The His-tagged fusion protein representing the extracellular domain of MOG (amino acids 1–125) was purified under denaturing conditions by metal chelate affinity chromatography on Ni-NTA Agarose columns (Qiagen) according to the manufacturer's guidelines.

Results

*Predicting the binding of MOG epitopes to HLA-DR4 (DRB1*0401) molecules*

To characterize the complement of MOG peptides that could bind to the human HLA-DR4 molecule and could therefore play a role in MS, we screened the MOG protein sequence with peptide binding algorithms which had been reported previously to predict the binding of peptides to HLA-DRB1*0401 (22, 23). These algorithms take into account the properties of the Ag binding site and of the amino acid side chains of a peptide to predict its binding to MHC molecules (25). According to the method developed by Hammer et al. (22), a numeric value is generated for all 9-mer amino acid sequences of a given protein, and only peptides with binding scores greater than 2 are expected to bind to HLA-DRB1*0401. The results for the screening of the extracellular region of the MOG protein are summarized in Table I. Of these, the sequence MOGp99–107 (FFRDHSYQE) was the peptide with the highest binding score (3.9). This peptide has an aromatic amino acid (phenylalanine) at the anchor position P1, and an amino acid with a hydroxyl group (serine) at position P6, consistent with optimal binding to HLA-DRB1*0401 (26–28). Several other peptides achieved predicted binding scores above 2, such as MOGp83–91 (LRIRNVRFS), which has an aliphatic anchor at P1 (leucine) and a valine at position P6 of the sequence, and was scored at 2.8. These results were confirmed overall when compared with a second binding algorithm developed by Marshall et al. (23). However, the predictions conflicted for some of the peptides. For example, MOGp83–91 had a good binding score of 2.8 with the Hammer method (22) but had an IC_{50} of 17.83 with the Marshall algorithm (23), indicative of low-affinity binding. Furthermore, the Hammer algorithm (22) predicted several peptides to be nonbinders for HLA-DRB1*0401, which were, however, estimated to be high-affinity binders by the Marshall method (Ref. 23 and data not shown). Hence, the binding algorithms alone were not sufficient to unambiguously determine the complement of MOG sequences that could be targeted by T cells in HLA-DR4$^+$ MS patients.

Table I. *Side chain scanning of MOG for HLA-DR4 (DRB1*0401) binding sequences*[a]

Amino Acid Position	MOG Peptide Sequence	Binding Score	Predicted IC_{50} (μM)
99–107	FFRDHSYQE	3.9	0.8
15–23	LVGDEVELP	3.0	1.18
83–91	LRIRNVRFS	2.8	17.83
120–128	YWVSPGVLV	2.5	0.09
40–48	YRPPFSRVV	2.1	14.5

[a] Scanning of the MOG sequence and calculation of peptide scores and estimated IC_{50} were performed as outlined in *Materials and Methods*. Shown are the results for the extramembraneous region of MOG (amino acids 1–125). Sequences with combined peptide binding scores above 2 were arranged according to their binding score (third column). Estimated IC_{50} values for the corresponding 13-mer MOG amino acid sequences are shown in the last column. Lower estimated IC_{50} indicate higher-affinity HLA-DRB1*0401 binding and are inversely proportional to peptide binding scores.

*Mapping of MOG epitopes in HLA-DR4 (DRB1*0401) transgenic mice*

To test whether or not the predicted and/or any of the other MOG epitopes were generated in vivo and induced T cell immunity, we used a transgenic mouse line that expressed HLA-DR4 (DRA-I-Eα/HLA-DRB1*0401-I-Eβ) molecules under the mouse MHC class II promoter (15). These transgenic mice are deficient for endogenous mouse MHC class II molecules, have no significant deletions in their T cell repertoire, and have previously been shown to mount strong T cell responses to various self and foreign Ags (15, 29).

As shown in Fig. 1, HLA-DR4 transgenic mice were immunized with the extracellular portion of MOG (rhMOG, amino acids 1–125), and T cell responses were measured in draining lymph node cells after recall with overlapping 20-mer MOG peptides by cytokine ELISPOT assay. Vigorous IFN-γ responses were detected after recall with the peptides MOGp91–110 and MOGp81–100, and occasionally with MOGp21–40. Furthermore, the mice mounted a vigorous Ag-specific IL-2, but not IL-5, recall response to these peptides (data not shown), consistent with the induction of Th1 immunity. None of the other MOG peptides induced significant cytokine production. The same MOG determinant hierarchy was seen when the mice were tested in parallel in proliferation assays for recall responses to the overlapping MOG peptides (data not shown). Mice not immunized, or immunized with irrelevant control Ags (e.g., hen egg white lysozyme, MBP), showed no responses to any of the MOG peptides (data not shown), demonstrating the specificity of the T cell response.

The minimal core epitope of the immunodominant region 91–110 of MOG peptide was comprised of amino acids 99–107, as determined by recall with overlapping MOG peptides shifted by one amino acid covering the region amino acids 90–110 (Fig. 1*B*). Cell separation for CD4 and CD8 T cells demonstrated that the observed T cell responses were mediated by CD4$^+$, but not by CD8$^+$, T cells (data not shown).

Taken together, the data showed that MOGp99–107 was the immunodominant HLA-DR4-restricted MOG peptide, and MOGp80–91 and MOGp21–40 were subdominant determinants. The results correlated well with the binding predictions (Table I) and demonstrate that these peptides were processed and presented in vivo after immunization with the rhMOG protein. Interestingly, not all of the predicted peptides induced T cell responses in vivo. These epitopes may either not be processed, or may not bind well enough to HLA-DRB1*0401 to be presented. There may be other mechanisms preventing T cell responses for these determinants, such as tolerance at the T cell level.

*In vitro binding of the region MOGp99–107 (FFRDHSYQE) to HLA-DR4 (DRB1*0401)*

The data on the predicted binding and ex vivo T cell reactivity suggested that the region MOGp99–107 had a high affinity for the HLA-DR4 molecule. However, to formally determine the binding affinity of this region, purified HLA-DRB1*0401 molecules were incubated with a biotinylated reference peptide (CLIP, 250 nM), and serial dilutions of the respective overlapping 9-mer peptides (100–0.001 μM) covering amino acids 91–108 of the MOG sequence were added. As shown in Fig. 2, this region of the MOG sequence indeed bound with a high affinity to HLA-DR4, as demonstrated by IC_{50} values of 4, 0.07, and 7.4 μM for the peptides MOGp97–105 (TCFFRDHSY), MOGp98–106 (CFFRDHSYQ), and MOGp99–107 (FFRDHSYQE) respectively. Thus, the in vitro binding data confirmed the algorithm prediction.

Induction of EAE with MOG peptides in HLA-DR4 transgenic mice

Several HLA-DR4 (DRB1*0401)-restricted MOG epitopes, including the region MOGp91–110 (core 99–107, FFRDHSYQE), induced T cell immunity in HLA-DR4 (DRB1*0401) transgenic mice. To establish whether or not these MOG epitopes played a role in the induction of EAE, we immunized the HLA-DR4 transgenic mice with MOGp97–108 (TC***FFRDHSYQE***E) and observed the mice for clinical and histological signs of EAE (Fig. 3*A*,

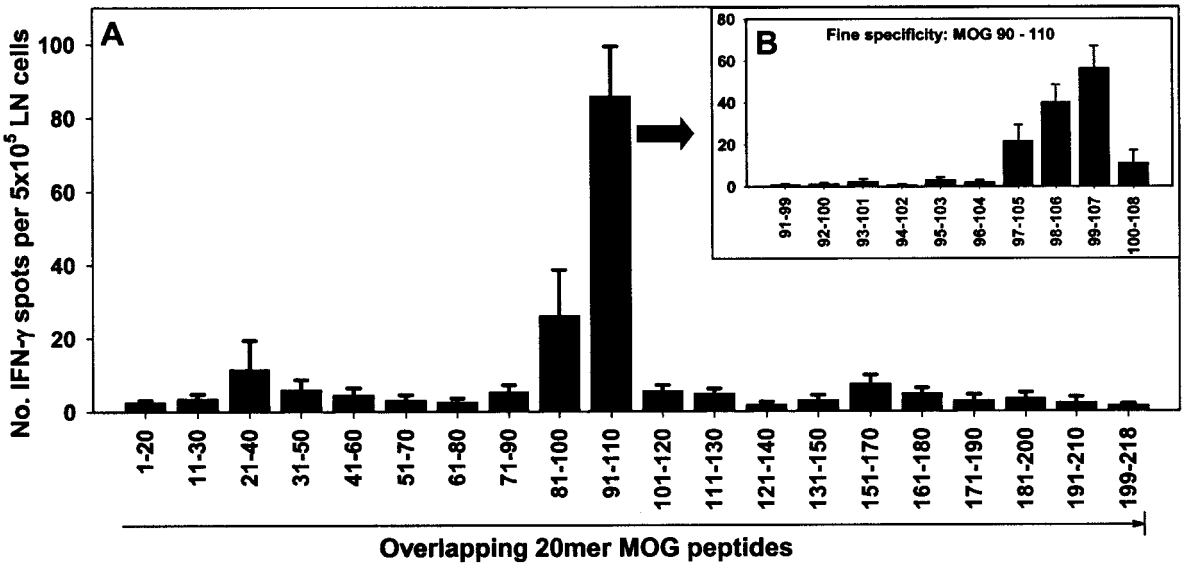

FIGURE 1. Mapping of HLA-DR4 (DRB1*0401)-restricted MOG epitopes. Six- to 10-wk old HLA-DR4 (DRB1*0401) transgenic mice were immunized with rhMOG (amino acids 1–125) in CFA s.c. Ten days later, frequencies of Ag-specific IFN-γ-producing T cells were measured by cytokine ELISPOT assay in single cell suspensions of draining lymph node cells. *A*, Shown are the mean and SE of the frequencies of MOG peptide-specific IFN-γ spots per 5×10^5 cells after recall with overlapping 20-mer MOG peptides spanning the whole MOG sequence ($n = 12$ mice). *B*, Frequencies of MOG peptide-specific IFN-γ spots per 5×10^5 cells after recall with overlapping 9-mer peptides shifted by one amino acid covering the region MOG amino acids 90–110 ($n = 6$ mice). The means were calculated from the results of triplicate wells, with the background subtracted (usually fewer than five spots), obtained in three independent experiments. Stimulation index of greater than 3 are considered positive as outlined in *Materials and Methods*.

●). Interestingly, immunization of the transgenic mice with this peptide resulted in severe EAE at 8–14 days after immunization. Brain sections of mice with EAE stained by H&E showed perivascular and periventricular inflammatory infiltrates consisting of lymphocytes, macrophages, and occasional neutrophils (Fig. 4, *A* and *B*). Immunofluorescence staining of brain sections with mAbs demonstrated that the infiltrates consisted of macrophages/microglia (MAC-1^+, F4/80^+) and $CD4^+$ T cells (Fig. 4, *C* and *D*). No B cells or $CD8^+$ T cells were detected (data not shown). Furthermore, immunization of the transgenic mice with the subdominant MOG epitopes MOGp21–40 or MOGp80–91 also induced EAE in the HLA-DR4 transgenic mice (Fig. 3*B*, □ and ▲, respectively), demonstrating that these determinants were encephalitogenic.

Taken together, the results show that EAE could be induced in the transgenic mice by immunization with HLA-DR4-restricted MOG epitopes.

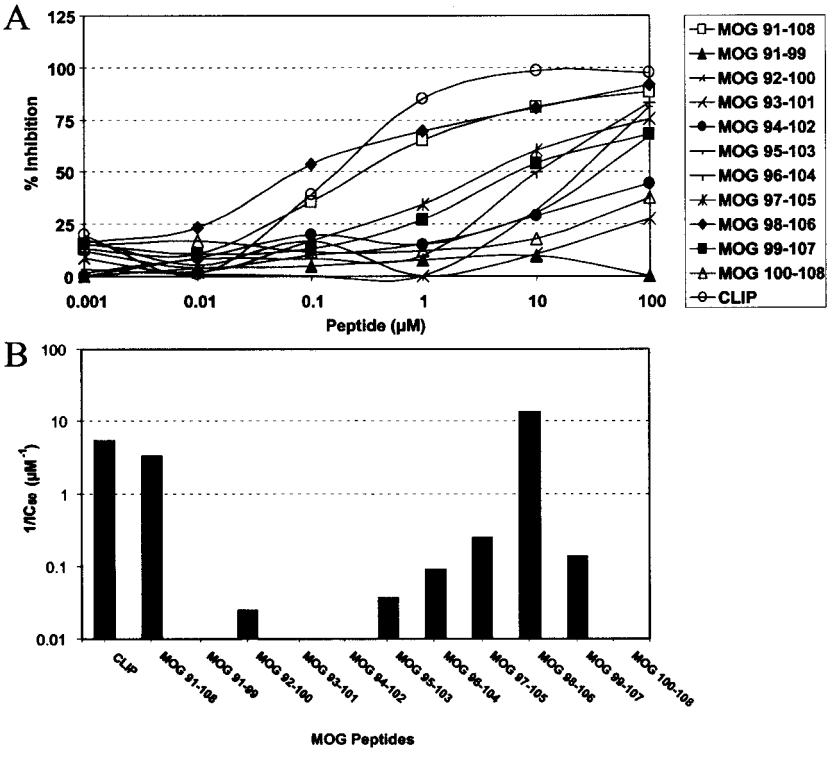

FIGURE 2. In vitro binding affinity of MOGp91–108 peptides to HLA-DR4 (DRB1*0401) molecules. Overlapping 9-mer peptides of the region MOGp91–108 were probed for their binding to purified HLA-DR4 (DRB1*0401) molecules, and IC_{50} values for each of the peptides were derived from the inhibition curves obtained by ELISA as described in *Materials and Methods*. *A*, Inhibition of the binding of the biotinylated tracer peptide CLIP by MOG peptides. *B*, Inverse IC_{50} of the respective MOG peptides.

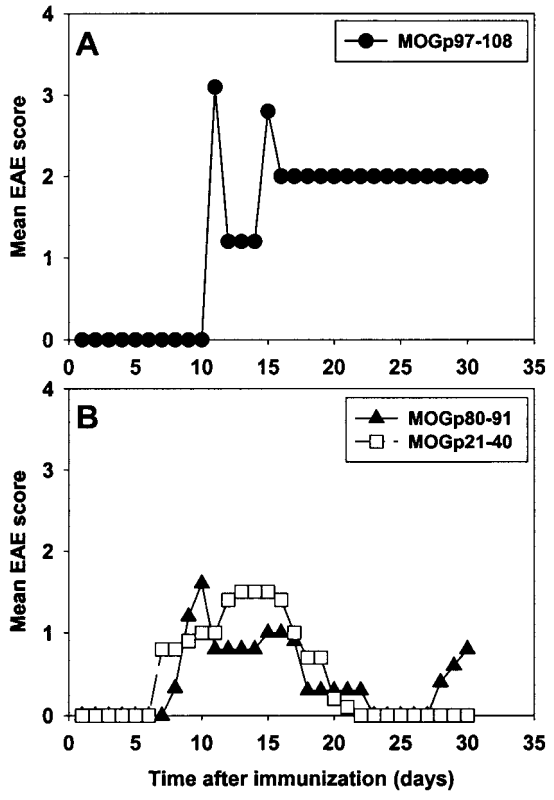

FIGURE 3. MOGp97–108 induces EAE in HLA-DR4 (DRB1*0401) transgenic mice. Transgenic mice were immunized with MOG peptides in CFA and pertussis toxin. Mice were observed daily for clinical signs of EAE and scored accordingly (see *Materials and Methods*). Shown is a representative experiment of six experiments performed. *A*, HLA-DR4 transgenic mice immunized with MOGp97–108 (●, $n = 10$). *B*, HLA-DR4 transgenic mice immunized with MOGp21–40 (□, $n = 6$) or MOGp80–91 (▲, $n = 5$) respectively.

Human B cells process and present the immunodominant MOG epitope

To test whether or not human APCs could present the encephalitogenic MOG peptides, we purified $CD4^+$ T cells from MOGp97–108-immunized transgenic mice and stimulated them with Ag and a human HLA-DR4 homozygous B cell line as APCs (16). As shown in Fig. 5, the human B cells, pulsed either with the MOGp97–108 peptide or with the whole rhMOG protein, induced vigorous cytokine production by the MOGp97–108-specific T cells (filled bars). No T cell response was induced with the irrelevant control peptide, human collagen type II p261–273. In contrast, hCIIp261–273-specific $CD4^+$ T cells (gray bars) responded to the hCII peptide, but not to MOG or the MOG peptide. Furthermore, fixing of the HLA-$DR4^+$ human B cells in 1% paraformaldehyde completely abrogated the induction of T cell responses when pulsed with the MOG protein, whereas the fixed cells induced strong T cell responses to the MOGp97–108 (data not shown). Similarly, HLA-$DR4^-$ B cells failed to induce specific T cell responses to either the MOG protein or the MOG peptide (data not shown).

Taken together, the data show that human APCs processed and presented the immunodominant HLA-DR4-restricted MOG peptide that we have identified in the transgenic mice. Therefore, the data suggest that this MOG peptide could also play a role in the pathogenesis of human MS.

Discussion

We have characterized for the first time the human MOG epitopes presented in the context of HLA-DR4 (DRB1*0401). The observation that the T cell response was directed against MOG peptides with high binding affinity for HLA-DR4 may reflect the notion that this self-Ag is not expressed in the thymus (30, 31). Thus, MOG-specific T cells are not negatively selected and express high-avidity TCRs for the MHC:peptide complex. How profoundly thymic negative selection is affected by the expression (or lack thereof) of myelin Ags in the thymus has recently been demonstrated for MBP

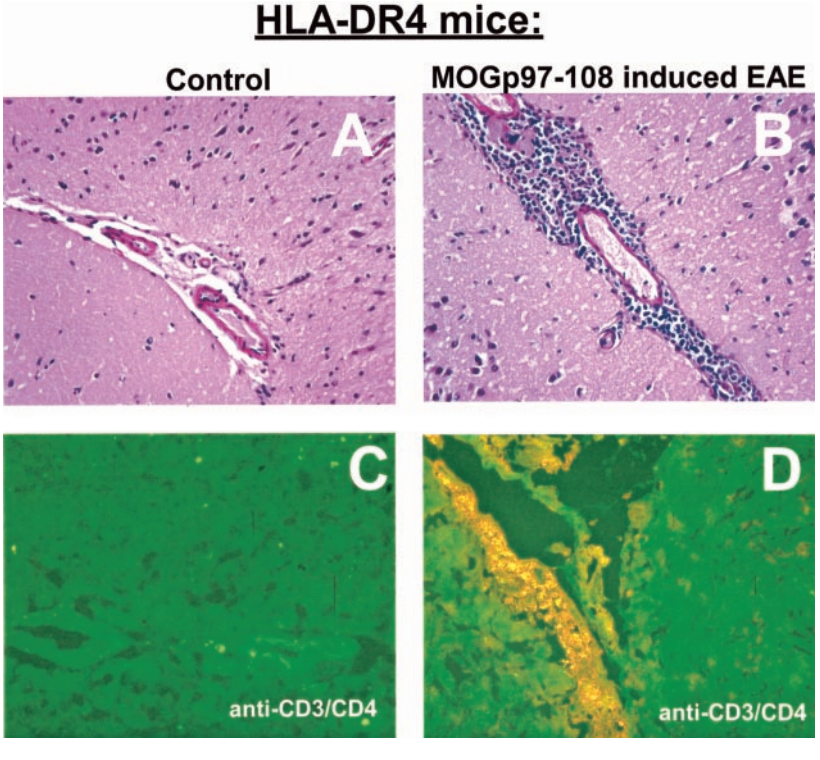

FIGURE 4. MOGp97–108-induced EAE is characterized by $CD4^+$ T cell infiltrates in the CNS. Histologic analysis of brain sections of representative H&E-stained (*A* and *B*) HLA-DR4 (DRB1*0401) transgenic mice was performed as outlined in *Materials and Methods*. *A*, Unimmunized HLA-DR4 transgenic control mouse without EAE. No inflammation is present. *B*, Representative MOGp97–108-immunized HLA-DR4 mouse with EAE shows extensive perivascular inflammatory infiltrates. *C* and *D*, Immunofluorescence microscopy of the same animals was performed as outlined in *Materials and Methods*. Shown in *D* is a representative section of anti-CD3-PE/CD4-FITC-stained brain from HLA-DR4 transgenic mice with MOGp97–108-induced EAE. Extensive infiltrates consisting of $CD3^+CD4^+$ T cells are present. MAC-1^+ cells were also present, but no $CD8^+$ or $CD19^+$ cells were detected (not shown).

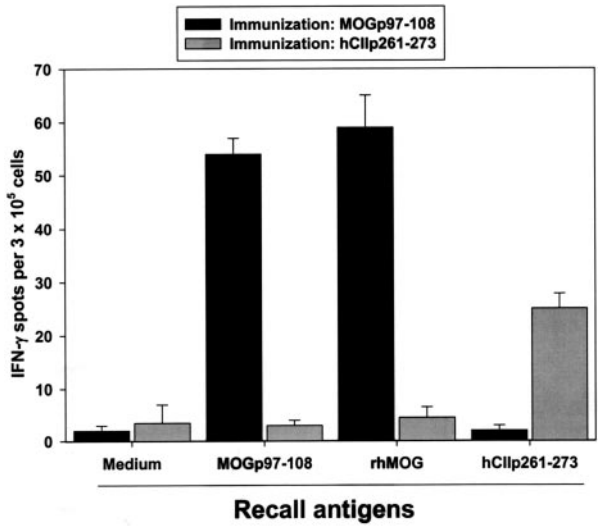

FIGURE 5. Human B cells activate MOGp97–108-specific $CD4^+$ T cells. HLA-DR4 (DRB1*0401) transgenic mice were immunized with MOGp97–108 (filled bars) or the human collagen type II peptide p261–273 (gray bars). Ten days later, draining lymph node cells were isolated and pooled, and $CD4^+$ T cells were purified by column separation as outlined in *Materials and Methods*. The purified $CD4^+$ T cells were tested in IFN-γ ELISPOT assays using a human B cell line homozygous for HLA-DR4 (DRB1*0401) as APCs as outlined in *Materials and Methods*. Shown is a representative experiment of four experiments performed.

and PLP (32–35). However, there are alternative mechanisms by which myelin-specific T cells could escape negative selection. For example, the immunodominant MBP epitope Ac1–11 is expressed in the thymus of B10.PL mice. However, MBP Ac1–11 is an extremely weak $H\text{-}2^u$ binder and forms unstable MHC:peptide complexes that fail to negatively select T cells (36, 37). This may explain why a documented immunodominant MBP peptide in $HLA\text{-}DR4^+$ MS patients binds HLA-DR4 (DRB1*0401) with relatively low affinity (13). In contrast, the immunodominant MOG region 97–108 is a high-affinity binder to HLA-DR4 (Fig. 2). Hence, our results suggest that it is not sufficient to identify myelin epitopes as targets for the treatment of autoimmune diseases based on peptide binding predictions or in vitro binding assays. Confirmation of the relevance of the autoantigenic epitopes by in vivo studies is required, such as we have demonstrated in the HLA-DR4 transgenic mice.

Theoretically, myelin epitopes need to be presented in the CNS to activate encephalitogenic T cells. That this actually occurs in vivo has recently been shown for MBPp84–102 in the context of HLA-DR2 (38). However, MBP is an abundant Ag in the CNS, whereas MOG comprises only 0.01–0.05% of the myelin mass (39). Therefore, it is not known whether it is processed and presented in the CNS, and by which type of APCs. It is particularly interesting that in our studies, MOGp97–108-reactive T cells induced EAE, suggesting that this peptide was presented in the CNS. Furthermore, the complete sequence homology of this region with human MOG (and >90% homology for the other MOG peptides identified) suggested that this peptide could similarly be presented in the CNS of MS patients and play a role in the pathogenesis of this disease. The significance of MOG for the autoimmune process is supported by the observation that MOG-reactive T cells are readily detectable in MS patients (40, 41), and MOG immunization induced severe EAE in a non-human primate model of MS (42).

The presented data confirm reports by other investigators that Ag processing and presentation by human and mouse APCs are sufficiently similar to permit the identification of antigenic epitopes within foreign- and self-Ags based on the binding properties of the MHC molecule (43, 44). Furthermore, by using in our studies transgenic mice that expressed HLA-DRA-I-Eα/HLA-DRB1*0401-I-Eβ molecules under the control of the mouse MHC class II promoter (15), it was ensured that T cell maturation and Ag recognition in these mice were unaffected. Thus, the TCR repertoire of the transgenic mice had no significant deletions, and hence there was enough plasticity of the T cell repertoire to detect the MOG epitopes presented in the context of HLA-DRB1*0401 (45). Most importantly, human B cells pulsed with the MOG protein activated MOGp97–108-reactive T cells (Fig. 5), demonstrating that this peptide could be naturally processed and presented to high-avidity T cells in HLA-DR4-expressing MS patients.

If MOG-reactive T cells have a high avidity for their Ag (experiments are under way in our laboratory to formally test for this), then it may be easier to activate the naive T cell precursors, by molecular mimicry, for example. Furthermore, the frequency of MOG-reactive T cells necessary to induce MS may be very low, perhaps even beyond the detection limit of current assays. Subsequently, T cells specific for other myelin Ags (MBP, PLP) or bystander T cells (specific for unrelated Ags) may cross the blood-brain barrier and perpetuate the MOG-induced disease process.

In summary, we have defined for the first time the nature of the MOG T cell epitopes restricted by human HLA-DR4. Our results show that the HLA-DR4 molecule selected for T cell responses to a high-affinity MHC binding peptide. This peptide was encephalitogenic and was also processed and presented by human APCs. Thus, the identified MOG epitopes are potential targets for Ag-specific immunotherapy.

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Publikation 6

MHC Class II Isotype- and Allele-Specific Attenuation of Experimental Autoimmune Encephalomyelitis[1]

Katrien L. de Graaf,* Silvia Barth,* Martin M. Herrmann,* Maria K. Storch,‡ Christoph Otto,§ Tomas Olsson,¶ Arthur Melms,* Günther Jung,†‖ Karl-Heinz Wiesmüller,†‖ and Robert Weissert[2]*

Most autoimmune diseases are associated with certain MHC class II haplotypes. Autoantigen-based specific immune therapy can lead either to beneficial or, in the context of inflammatory conditions, detrimental outcomes. Therefore, we designed a platform of peptides by combinatorial chemistry selected in a nonbiased Ag-independent approach for strong binding to the rat MHC class II isotype RT1.D^n allelic product of the RT1^n haplotype that is presenting autoantigen in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in LEW.1N rats. Peptide p17 (Ac-FWFLDNAPL-NH$_2$) was capable of suppressing the induction of and also ameliorated established experimental autoimmune encephalomyelitis. MHC class II isotype and allele specificity of the therapeutic principle were demonstrated in myelin basic protein-induced experimental autoimmune encephalomyelitis in LEW rats bearing the RT1^l haplotype. A general immunosuppressive effect of the treatment was excluded by allogeneic heart transplantation studies. In vitro studies demonstrated the blocking effect of p17 on autoantigenic T cell responses. We thus demonstrate a rational design of strong MHC class II-binding peptides with absolute isotype and allele specificity able to compete for autoantigenic sequences presented on disease-associated MHC class II molecules. ***The Journal of Immunology*****, 2004, 173: 2792–2802.**

Most autoimmune diseases are associated with certain MHC class II isotype allelic variants (1). Without much doubt, this is due to presentation of autoantigenic peptides on certain MHC class II molecules (2). A self-reactive T cell repertoire reactive with the presented peptide fragments must be available for development of autoimmunity (3). Theoretically, treatment of autoimmune diseases in an immunoselective manner would be possible by targeting particular allelic variants of MHC class II isotypes. But, dependent on the circumstances, autoantigen-based specific immunotherapy can lead to a beneficial or a detrimental outcome (4, 5). The outcome is dependent on the individual T cell repertoire and inflammatory stimuli. Altered peptide ligands use an autoantigenic peptide with minor alterations compared with the native self peptide. Such therapy has worsened disease in a number of multiple sclerosis (MS)[3] patients (6). Most likely, this is due to the capacity of an altered peptide ligand to function as antagonist, agonist, and superagonist, depending on the particular TCR (7). Targeting of dendritic cells with autoantigen can lead to tolerance (4). This can be reverted in the context of inflammation in which dendritic cells can present autoantigen in such a way that autoimmunity is induced (5, 8). Therefore, a selective targeting of certain allelic variants of MHC class II isotypes with sequences without any structural similarity to self Ags would possibly be a way to prevent unwanted autoimmunity.

MS is an inflammatory disease of the CNS with demyelination and axonal and neuronal loss (9). Currently, available treatments are only modestly effective and there is need for improved therapies that could stop further disease development. MS is a complex genetic disease that is strongly associated with the isotypes and alleles HLA-DR2a, DR2b, and DQ6 in U.S. Americans and Northern Europeans (10). Understanding of immunogenetic mechanism governed by MHC genes may be studied in rodent models of MS. Hereby, inbred rat strains induced to develop experimental autoimmune encephalomyelitis (EAE) are important tools. Susceptibility or resistance in many of these models is associated with the MHC (RT1 in the rat) class II gene products (11). This is not surprising in view of the key role of MHC class II molecules in triggering CD4$^+$ T cells by presenting restricted sets of peptides to the TCR. Little is known about the structural characteristics of rat MHC class II molecules. Currently, only the RT1.B^l ligand-binding motif, an HLA-DQ-like molecule of the LEW (RT1^l) rat, has been described (12, 13). Combinatorial peptide libraries have been successfully applied in the past to examine different aspects of MHC class I and II molecule interactions with peptide (14, 15).

*Experimental Neuroimmunology Laboratory, Department of General Neurology, Hertie Institute for Clinical Brain Research, and †Department of Organic Chemistry, University of Tübingen, Tübingen, Germany; ‡Department of Neurology, University of Graz, Graz, Austria; §Experimental Transplantation Immunology, Department of Surgery, University of Würzburg, Würzburg, Germany; ¶Neuroimmunology Unit, Center for Molecular Medicine, Karolinska Hospital, Stockholm, Sweden; and ‖EMC Microcollections, Tübingen, Germany

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[2] Address correspondence and reprint requests to Dr. Robert Weissert, Experimental Neuroimmunology Laboratory, Department of General Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen, Hoppe-Seyler-Strasse 3, 72076 Tübingen, Germany. E-mail address: robert.weissert@uni-tuebingen.de

[3] Abbreviations used in this paper: MS, multiple sclerosis; COP-1, copolymer-1; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MNC, mononuclear cell; MOG, myelin oligodendrocyte glycoprotein; p.i., postimmunization; rrMOG, rat rMOG.

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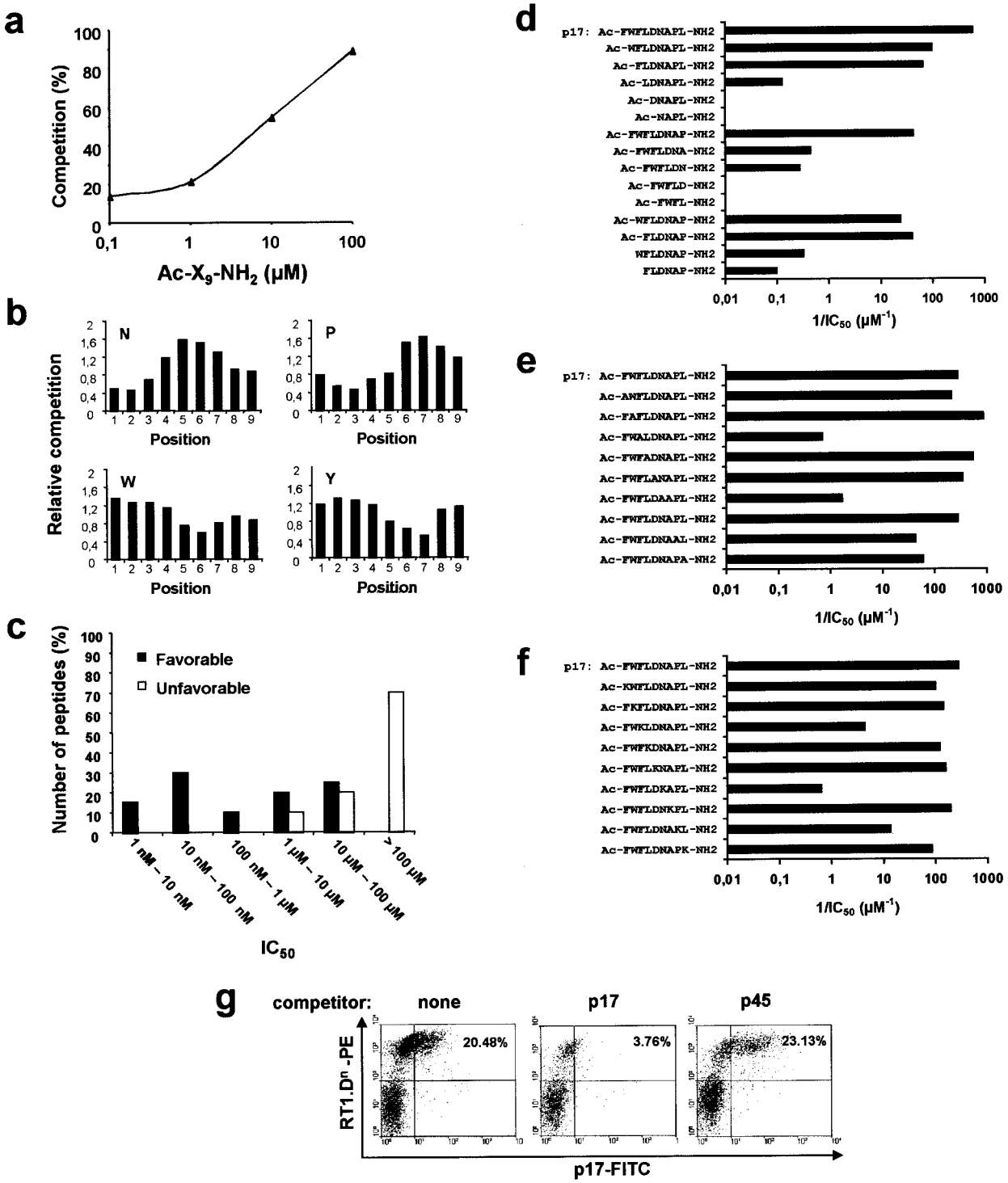

FIGURE 1. Binding of Ac-X_9-NH_2 acetylated amide sublibraries and predicted peptides to RT1.D^n. *a*, The competition of Ac-X_9-NH_2 with biotinylated CLIP 97–120 for binding to purified RT1.D^n molecules was measured. *b*, The competition of acetylated nonapeptide amide sublibraries with biotinylated CLIP 97–120 for binding to purified RT1.D^n molecules was assessed. The relative competition (= percentage of competition of O_x/mean percentage of competition of $O_{1–9}$) of 4 defined aa (N, P, Y, and W) in their respective sequence positions is shown. *c*, The competition of predicted peptides (Table II) with biotinylated CLIP 97–120 for binding to purified RT1.D^n molecules was analyzed. *d*, Binding of truncated p17 variants. *e*, Binding of alanine (A)-substituted p17 variants. *f*, Binding of lysine (K)-substituted p17 variants. *g*, Binding of p17-FITC to intact B cells. p17-FITC was competed out with unlabeled p17 or p45.

Such libraries offer the distinct advantage of being able to quantitatively assess the contribution of each amino acid residue in each position for interaction with the peptide-binding groove of the analyzed MHC class II isotype (RT1.B and RT1.D) allelic variants.

The extracellular domain of myelin oligodendrocyte glycoprotein (MOG 1–125) and its encephalitogenic core sequence, MOG 91–108, induce a very MS-like lesional spectrum in the CNS with inflammation, demyelination, and axonal loss in LEW.1N (RT1^n) rats (16–18). Therefore, we used this model for our investigations.

Materials and Methods

Peptide libraries and peptides

Synthetic acetylated nonapeptide amide libraries, as well as defined acetylated nonapeptide amides and biotinylated peptide amides were prepared by fully

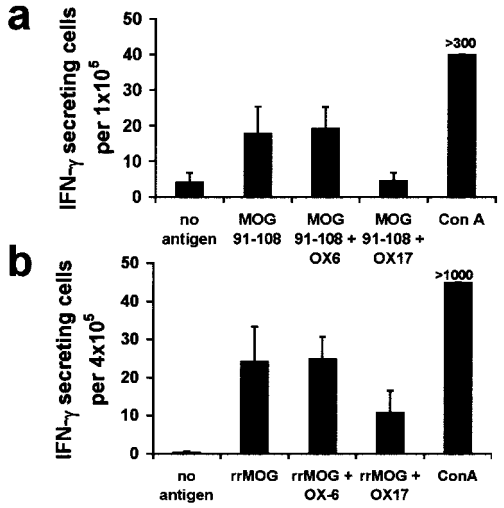

FIGURE 2. MHC restriction of the encephalitogenic T cell response. *a*, The intracerebral encephalitogenic MOG 91–108-specific T cell response in LEW.1N (RT1^n) rats is RT1.D^n restricted, as was demonstrated by inhibition of the T cell response by addition of the mAb OX-17 (anti-RT1.D) as compared with mAb OX-6 (anti-RT1.B) ($n = 4$). *b*, Similar results were obtained for the peripheral T cell response against MOG 1–125 in LEW.1N (RT1^n) rats that was RT1.D^n restricted as well ($n = 4$).

automated solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl/*tert*.-butyl (Fmoc/tBu) chemistry and analyzed by HPLC and electrospray ionization mass spectrometry (19). Biotinylated CLIP peptide 97–120 (LPKSAK-PVSPMRMATPLLMRPSMD) was obtained by elongating the peptide with two spacer amino acids, followed by biotin using a coupling method. p17-FITC was obtained from EMC Microcollections. Copolymer-1 (COP-1) was obtained from TEVA Pharma (Kirchzarten, Germany).

Purification of MHC molecules and peptide-binding studies

RT1.D^n, RT1.B^n, RT1.D^l, and RT1.B^l molecules were purified from thymic and splenic tissue from LEW.1N (RT1^n) rats and LEW (RT1^l) rats by affinity chromatography using the mAbs OX-17 (anti RT1.D) and OX-6 (anti RT1.B), as previously described (18). Binding assays were performed with a competitive ELISA based on a dissociation-enhanced lanthanide fluoroimmunoassay (Wallac, Turku, Finland) (18). For the competitive ELISA, a 50 nM solution of RT1.D molecules was incubated for 48 h at 37°C with 100 nM biotinylated CLIP 97–120 and 2 μM acetylated nonapeptide amide sublibrary or with various concentrations of competitor peptides. The concentration of totally randomized acetylated nonapeptide library Ac-X_9-NH_2 yielding 30% competition was used for measuring the competition with the acetylated nonapeptide amide libraries (Fig. 1*a*). Competitor peptides were measured at various concentrations ranging from 1 nM to 100 μM. The IC_{50} of a peptide was defined as the concentration of peptide necessary for the inhibition of binding of the tracer peptide by 50%.

Rat rMOG 1–125

Rat rMOG 1–125 (rrMOG 1–125) was expressed in *Escherichia coli* and purified by chromatography (16).

Rats, immunization protocol, and scoring of EAE

Rats were obtained from H. Hedrich (Central Animal Laboratory, Hannover Medical School, Hannover, Germany). They were bred and kept under specific pathogen-free conditions. Two to four rats were housed per cage and obtained food and water ad libitum. Female rats between age 8 and 10 wk were used for all experiments. All experiments were approved by the regional boards in Tübingen and Würzburg, Germany.

Rats were injected intradermally at the base of the tail either with 100 μg of MOG 91–108, myelin basic protein (MBP) 63–88, or MBP 85–99, or with 20 μg of rrMOG 1–125. For blocking experiments, 100 μg of p45, p17, p79, or COP-1 was added. The Ags in a total volume of 100 μl were mixed with 100 μl of CFA (1:1). A total of 100 μl of CFA consisted of IFA (Sigma-Aldrich, St. Louis, MO) and 200 μg (for rrMOG 1–125-induced EAE) or 500 μg (for MOG 91–108-induced EAE) of heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco Laboratories, Detroit, MI). For treatment of ongoing disease, LEW.1N (RT1^n) rats were immunized with 50 μg of rrMOG 1–125 in IFA. On day 11 postimmunization (p.i.), rats received a single injection of 500 μg of p45 or p17 in 500 μl of IFA i.p. Rats were examined daily for signs of EAE and weighed from day 7 p.i. until sacrifice. The clinical scoring was as follows: 0 = no illness; 1 = tail weakness or paralysis; 2 = hind leg paraparesis or hemiparesis; 3 = hind leg paralysis or hemiparalysis; 4 = tetraparesis or moribund; 5 = death.

Cellular assays and elution of cells from the CNS

Mononuclear cells (MNC) from draining lymph nodes, spleen, and CNS were obtained, as described (18). B cells from the spleens of naive rats were purified by magnetic cell separation against CD45RA (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, cytofluorometric analysis by FACS with anti-RT1.D mAb (Ox-17) and p17-FITC (1 μM) was performed. Competition experiments were performed with unlabeled p17 (200 μM) and p45 (200 μM).

Enumeration of IFN-γ-secreting cells by ELISPOT was performed, as described (18). To assess the MHC class II restriction of the generated IFN-γ responses, 10 μg/ml OX-6 (anti-RT1.B), OX-17 (anti-RT1.D), or Tib191 (isotype-matched control) was added to the cells together with the Ag.

CNS-infiltrating cells were eluted from the CNS of diseased rats by density gradient centrifugation, as described (18). A total of 1×10^4 cells was incubated with 1×10^5 irradiated (30 Gy) thymocytes with or without Ags. Specificity was assessed by ELISPOT for IFN-γ-secreting cells.

Determination of MOG-specific Abs

The determination of rrMOG 1–125-specific IgG was performed, as described (18). The OD was read at 405 nm.

cDNA synthesis and quantification of cytokine mRNA levels using real-time PCR

Quantitative real-time PCR and primer design were performed, as described (18). Relative quantity of mRNA levels was determined using the $\Delta\Delta$CT method. The amount of mRNA in each sample was calculated as the ratio between the amount of cytokine mRNA and the amount of GAPDH mRNA in this sample. For cells without Ag or Ag-restimulated cells, the cytokine/GAPDH ratio of the samples from the rrMOG 1–125-immunized rats was set to 1, and the ratio of the samples from the p17 or p17 and rrMOG 1–125-coimmunized rats was calculated relative to this sample.

Transfer of spleen cells

LEW.1N (RT1^n) rats were immunized with either 100 μg of p45 or p17 in 500 μg of CFA at the base of the tail. On day 9 p.i., spleen MNC were isolated and 10^7 cells were transferred i.v. into naive LEW.1N (RT1^n) rats that were subsequently immunized with 50 μg of rrMOG 1–125 in 200 μg of CFA.

Cardiac transplantation

LEW (RT1^l) rats served as recipients, and WF (RT1^u) rats as donors. Heterotopic cardiac transplantation was performed according to the method of Ono and Lindsey (20). The allograft function was monitored by daily transabdominal palpation of cardiac contractions. Graft rejection was considered as the complete cessation of palpable cardiac contractions, which was then confirmed histologically.

Table I. *Influence of defined amino acid residues on competitive binding to RT1.D^n*

Sequence Position	Favorable	Unfavorable
1	F H W Y	A N P V
2	F I R W Y	D H N P Q
3	F L R W Y	D G N P
4	D I L M N	E G P S V
5	D N S T	I P V Y W
6	D N P	F K L W Y
7	A N P Q	F L R W Y
8	H P	D L N Q W
9	G I L M V	H N W

Table II. *IC_{50} values of acetylated nonapeptide amides for binding to RT1.D^n and prediction of binding properties*[a]

Peptide No.	Sequence	Prediction[b]	IC_{50} (μM)
p17	F W F L D N A P L	+	0.002
p11	Y F W I D N P P I	+	0.004
p3	Y Y Y M S D Q P V	+	0.007
p2	W W R I N N A H L	+	0.011
p18	W Y W I N D P H V	+	0.016
p12	H W R M N D P H L	+	0.018
p6	W F Y N T D A H I	+	0.032
p14	W R L D N N Q P M	+	0.033
p10	W I F D N P A P G	+	0.071
p5	F I W D S N A P G	+	0.10
p20	H I W M T N N P G	+	0.24
p16	H F Y L T P Q P I	+	1.78
p13	F Y F L D P N P V	+	2.00
p4	H R F L N P P H M	+	2.99
p9	F R Y M D D N P M	+	3.76
p128	P P N S Y W L L N	−	5.47
p43	P D D G W F R N N	−	10.0
p15	Y I R N S D N H G	+	13.3
p8	H Y R J D N P P V	+	18.8
p129	V Q P V V W R N W	−	19.3
p7	Y W L L D P P P L	+	25.1
p1	F F L L D P N P I	+	50.1
p19	Y R L I D P Q H M	+	89.1
p41	N H G P W Y F Q H	−	>100
p42	A N P V W K W L W	−	>100
p45	N H P S P K Y L W	−	>100
p47	A D N V I Y L D N	−	>100
p48	P P P G V F R W W	−	>100
p50	V N N S I F F W N	−	>100
p127	N Q G P Y L F D H	−	>100

[a] All peptides are N-terminally acetylated and C-terminally amidated.

[b] The peptides marked "+" consist of randomly selected combinations of favorable amino acids, whereas the peptides marked "−" consist of randomly selected combinations of unfavorable amino acids, according to Table I.

Histopathology

Histological evaluation was performed on paraformaldehyde-fixed, paraffin-embedded sections of brains and spinal cords on days 14 and 19 p.i. (16, 17, 21). Paraffin sections were stained with H&E and Luxol fast blue to assess inflammation and demyelination. An inflammatory index was calculated from the number of perivascular inflammatory infiltrates of each rat on an average of 15 complete cross sections of spinal cord. The degree of demyelination was evaluated for brain and spinal cord sections separately and semiquantitatively described and scored (16, 17, 21).

Statistical analysis

Student's *t* test was used for normally distributed variables. When the data did not fulfill the criteria of being normally distributed, nonparametric statistics (Mann-Whitney *U* test) were used. Values of *p* were adjusted for multiple comparisons.

Results

MHC class II restriction of the MOG-specific T cells

To find selective isotype- and allele-specific MHC class II-binding peptides for the LEW.1N (RT1^n) rat, we had to assess the restriction of the encephalitogenic T cell response toward the main encephalitogenic determinant within MOG 1–125 in LEW.1N (RT1^n) rats, MOG 91–108. There is no detectable ex vivo T cell response toward MOG 91–108 from peripheral lymphoid tissue after EAE induction with rrMOG 1–125 or MOG 91–108 (18). Therefore, we eluted infiltrating cells from the CNS on day 12 p.i. ($n = 4$). A restriction analysis by ELISPOT of the T cell response to MOG 91–108 was performed by adding mAbs OX-6 (anti-DQ) or OX-17 (anti-DR) together with Ag. The MOG 91–108-specific T cell response was reduced to ~30% by OX-17 (anti-RT1.D) as compared with OX-6 (anti-RT1.B) (Fig. 2*a*). Next, we assessed the restriction of the rrMOG 1–125 T cell response in the periphery by blocking with the mAbs OX-6 or OX-17 ($n = 4$). The T cell response was reduced by addition of OX-17 (anti-RT1.D) to ~50%, but not by addition of the OX-6 (anti-RT1.B) (Fig. 2*b*). These experiments indicated that the RT1.D^n molecule is the restriction element of the encephalitogenic T cell response in rrMOG 1–125-induced EAE.

Competition of acetylated nonapeptide amide libraries for binding to RT1.D molecules

The peptide-binding pattern of the RT1.D^n molecule was assessed by using combinatorial peptide libraries. In a first step, a completely randomized acetylated nonapeptide amide library (Ac-X_9-NH_2; X: 19 L amino acids excluding cysteine (C)) was tested for its capacity to bind to RT1.D^n in a competition ELISA. The randomized library readily competed for binding to RT1.D^n, and addition of 100 μM Ac-X_9-NH_2 inhibited binding of the biotinylated tracer peptide (rat CLIP 97–120, LPKSAKPVSPMRMATPLLMRPSMD) to RT1.D^n up to 90% (Fig. 1*a*). Because Ac-X_9-NH_2 bound satisfactorily and because the use of longer combinatorial libraries would probably complicate the obtained data due to translational invariance, acetylated nonapeptide amide sublibraries were selected for carrying out the subsequent competition experiments (14).

A total of 171 nonapeptide sublibraries (9 sequence positions × 19 aa, excluding C) was screened in a competition ELISA to elucidate the binding patterns of the RT1.D^n molecule. A concentration of 50 nM RT1.D^n in combination with 100 nM biotinylated CLIP 97–120 and 2 μM of each sublibrary was applied for the assays. We compared the competition of the 171 sublibraries, each characterized by 1 defined aa shifted over the 9 different positions, by measuring the relative competition of this amino acid at all 9 positions (relative competition = percentage of competition of O_x/mean percentage of competition of O_{1-9}) (Fig. 1*b*). Because in some cases (for example, for F) the differences in relative competition values were not incisive, measurements were confirmed in at least two independent experiments (data not shown). Moreover, frequently, amino acid residues with similar structural properties (for example, the sublibraries with F, W, and Y in defined positions) clearly showed comparable tendencies in the relative competition values (Fig. 1*b*). Thus, the effect of each amino acid in its respective position was termed as favorable, indifferent, or unfavorable.

Aromatic amino acids on P1, P2, and P3 of the sublibraries favored binding to RT1.D^n (Table I). Contrarily, aromatic residues rather inhibited binding if present at P5–P9. Both D and N enhanced binding at P4, P5, and P6, whereas P showed significant competition at P6, P7, and P8. Residues with aliphatic side chains such as I, L, M, V, as well as G were favorable at P9, and I, L, and M were also well tolerated at P4.

Competition of acetylated and amidated peptides to RT1.D^n molecules

To test the generated database (Table I) for its predictive value for high and low affinity binders, two sets of peptides were synthesized: the first set of peptides consisted of randomly selected combinations of favorable amino acids for each of the nine positions. The second set of peptides was made up of randomly selected combinations of unfavorable amino acids for each of the nine positions. IC_{50} values were measured for all defined acetylated nonapeptide amides (Table II). All peptides belonging to the set of favorable peptides showed significant binding and ranged from very high affinity ligands (IC_{50} = 2 nM for p17) to low affinity

ligands (IC_{50} = 89 μM for p19). In contrast, 70% of the unfavorable peptides did not show any competition at all, even at peptide concentrations of 100 μM (Fig. 1*c*). The binding of p17 was RT1.D^n isotype and allele specific: p17 neither bound RT1.B^n molecules (IC_{50} > 100 μM), nor RT1.D^l (IC_{50} = 12 μM) and RT1.B^l (IC_{50} > 100 μM) molecules (Table III).

All shortened variants of p17 indicated lower binding capabilities compared with p17 (Fig. 1*d*). Truncated peptides from the C-terminal, from the N-terminal parts, as well as from the C- and N-terminal parts of p17 revealed a minimum sequence of 6 aa for binding. C-terminal truncations resulted in stronger loss of binding compared with N-terminal truncations (Fig. 1*d*). Binding was strongly improved by acetylation at the N-terminal part of the shortened peptides (Fig. 1*d*). Subsequently, we substituted all positions in the p17 peptide sequence by an alanine and lysine scan and found that binding to RT1.D^n was dramatically inhibited in case of substituting positions 3 and 6 (Fig. 1, *e* and *f*).

Binding of p17 to MHC class II RT1^n molecules on live B cells

To analyze whether live APCs could present p17 on MHC class II molecules, we purified B cells from spleen. These were purified and stained for anti-RT1.D-PE (mAb Ox-6) and p17-FITC on the cell surface (Fig. 1*g*). Binding of p17 to RT1.D^n-positive cells was 20.5% (arbitrary threshold level set). Coincubated unlabeled p17 in 200-fold excess reduced binding of p17-FITC to 3.8%. Coincubated unlabeled p45 in 200-fold excess did not reduce binding of p17-FITC with 23.1% positive cells.

Immunogenicity of high affinity ligands in LEW.1N (RT1^n) rats

To determine whether treatment of LEW.1N (RT1^n) rats with synthetic high affinity ligands could prime for T cell reactivity to the peptides, rats were immunized with the four peptides showing the lowest IC_{50} values (p2, p3, p11, and p17) (n = 3 for each peptide). On day 12 p.i., the recall responses to these peptides in lymph node suspensions were evaluated by measuring the IFN-γ secretion using an ELISPOT assay (Fig. 3*a*). To test the restriction of the response, mAbs OX-6 (anti-RT1.B), OX-17 (anti-RT1.D), or Tib 191 (an isotype-matched control) were added to the culture medium (Fig. 3*b*) (n = 4 each peptide). The four different peptides raised striking IFN-γ responses, indicating that all investigated peptides were immunogenic and represented T cell agonists. As shown by the blocking experiments with mAb against RT.1B and RT1.D molecules, the responses elicited to the peptides were RT1.D restricted (Fig. 3*b*). The number of IFN-γ-secreting spots for the tested peptides did not clearly correlate to the IC_{50} values.

Amelioration of MOG-EAE in LEW.1N (RT1^n) rats

Next, the newly defined synthetic nonapeptides were investigated for their capacity to inhibit the induction of EAE in vivo. Because p17 (Ac-FWFLDNAPL-NH_2) had the lowest IC_{50} value for binding to RT1.D^n and therefore the highest affinity for the RT1.D^n molecule (Table II), this peptide was selected for testing its ability to inhibit MOG 91–108- and rrMOG 1–125-induced EAE in LEW.1N (RT1^n) rats in which the encephalitogenic response to

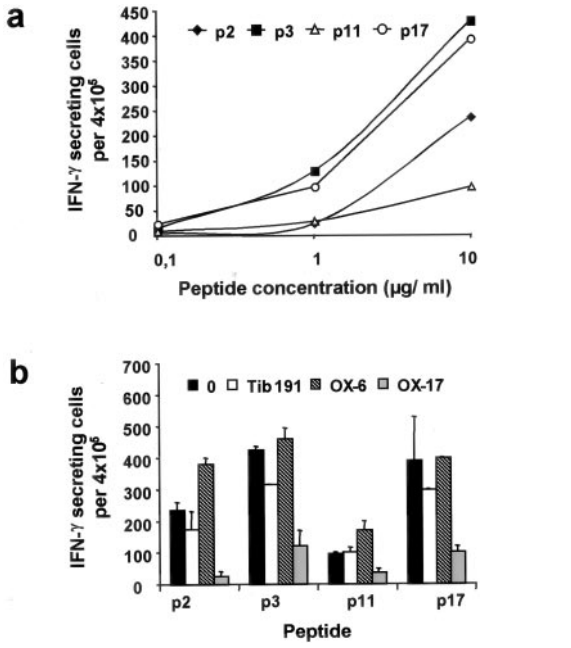

FIGURE 3. Immunogenicity and restriction of T cell responses to high affinity ligands. *a*, LEW.1N (RT1^n) rats were immunized with the high affinity-binding peptides p2, p3, p11, and p17, and T cell responses were assessed by IFN-γ ELISPOT (each n = 3). *b*, To test the restriction of the peptide responses, mAbs OX-6 (anti-RT1.B), OX-17 (anti-RT1.D), or Tib 191 (isotype matched, specific for irrelevant control Ag) were added to the cell cultures (each n = 4).

MOG 91–108 is RT1.D^n restricted (Fig. 2). Nonapeptide p45 (Ac-NHPSPKYLW-NH_2) was used as a control, because this was one of the peptides lacking any measurable affinity for RT1.D^n (IC_{50} > 100 μM; Table II).

First, LEW.1N (RT1^n) rats were immunized with the encephalitogenic MOG 91–108 peptide in CFA mixed with PBS alone (n = 8), p45 (n = 11), or p17 (n = 11) in PBS. Coadministration of p17 together with MOG 91–108 significantly prevented MOG 91–108-induced EAE compared with PBS alone ($p < 0.001$, sum score, t test) or p45 ($p < 0.001$, sum score, t test) (Table IV, group 1). None of the p17-treated rats developed any clinical signs of EAE.

Second, LEW.1N (RT1^n) rats were coimmunized with rrMOG 1–125 in CFA mixed with either p45 (n = 4), p17 (n = 12), or PBS alone (n = 8). Peptide p17 strongly ameliorated EAE in rrMOG 1–125-immunized rats compared with p45 ($p < 0.001$, sum score, t test) or PBS alone ($p < 0.001$, sum score, t test) (Table IV, group 2). To test whether competition at the level of MHC class II binding was the only mechanism responsible for EAE attenuation, LEW.1N (RT1^n) rats were injected with p17 and rrMOG 1–125 in CFA at different sites of the body (immunization with rrMOG 1–125 at tail base, p17 application in CFA at the neck). This treatment also resulted in amelioration of EAE (p17

Table III. *IC_{50} values of acetylated nonapeptide amides (p17 and p79) for binding to RT1.D^n, RT1.B^n, RT1.D^l, and RT1.B^l*

Peptide	Binding to RT1 Isotype and Allele			
	RT1.D^n	RT1.B^n	RT1.D^l	RT1.B^l
p17: Ac-FWFLDNAPL-NH_2	2 nM	>100 μM	12 μM	>100 μM
p79: Ac-FWYIAIQDE-NH_2	>100 μM	>100 μM	57 nM	>100 μM

Table IV. *Disease course of rats treated with high affinity ligands*

Group	Strain	Disease Induction	Adjuvants	Treatment	Day of Treatment	*n*	Onset of Symptoms (day)	Follow-up (days)	Incidence (%)	Maximum Score	Sum Score	*p* Sum Score
1a	LEW.1N	MOG 91–108 (100 μg)	CFA (200 μg)	PBS	0	8	10	27	75	1.4	19.1	
1b	LEW.1N	MOG 91–108 (100 μg)	CFA (200 μg)	p45[a] (100 μg)	0	11	10	27	81	1.7	21.4	
1c	LEW.1N	MOG 91–108 (100 μg)	CFA (200 μg)	p17[a] (100 μg)	0	11	None	27	0	0	0	$p < 0.001$ against 1a or 1b
2a	LEW.1N	rrMOG 1–125 (50 μg)	CFA (200 μg)	PBS	0	8	10	15	100	4.1	18.1	
2b	LEW.1N	rrMOG 1–125 (50 μg)	CFA (200 μg)	p45[a] (100 μg)	0	4	10	15	100	4.75	16.7	
2c	LEW.1N	rrMOG 1–125 (50 μg)	CFA (200 μg)	p17[a] (100 μg)	0	12	10	15	25	0.83	2.5	$p < 0.002$ against 2a or 2b
2d	LEW.1N	rrMOG 1–125 (50 μg)	CFA (200 μg)	p17[b] (100 μg)	0	8	10	15	100	3	6	$p < 0.001$ against 2a or 2b
2e	LEW.1N	rrMOG 1–125 (50 μg)	CFA (200 μg)	p17[a] dimer (100 μg)	0	10	None	40	0	0	0	$p < 0.001$ against 2a or 2b
3a	LEW.1N	rrMOG 1–125 (50 μg)	IFA	p45[a] (100 μg)	11	10	10	40	90	2.6	71.7	
3b	LEW.1N	rrMOG 1–125 (50 μg)	IFA	p17[a] (100 μg)	11	17	10	40	41	1.4	26.8	$p = 0.002$ against 3a
4a	LEW	MBP 85–99 (100 μg)	CFA (200 μg)	PBS	0	5	12	24	100	3	9.75	
4b	LEW	MBP 85–99 (100 μg)	CFA (200 μg)	p79[a] (100 μg)	0	5	12	24	40	1	3.5	$p = 0.007$ against 4a
5a	LEW	MBP 63–88 (100 μg)	CFA (200 μg)	PBS	0	5	10	24	100	2.7	10.5	
5b	LEW	MBP 63–88 (100 μg)	CFA (200 μg)	p79[a] (100 μg)	0	5	11	24	100	2.3	9.16	NS against 5a
6a	LEW	MBP 85–99 (100 μg)	CFA (200 μg)	PBS	0	5	12	24	100	2.4	14.4	
6b	LEW	MBP 85–99 (100 μg)	CFA (200 μg)	p17[a] (100 μg)	0	5	12	24	100	2.2	14	NS against 6a
7a	LEW	MBP 63–88 (100 μg)	CFA (200 μg)	PBS	0	5	12	24	100	3	17	
7b	LEW	MBP 63–88 (100 μg)	CFA (200 μg)	p17[a] (100 μg)	0	5	11	24	100	2.8	16.6	NS against 7a

[a] Site of immunization.
[b] Different site to immunization site.

treatment at different site compared with PBS control, $p < 0.002$, sum score, *t* test) (Table IV, group 2). Due to extreme disease severity present in LEW.1N (RT1^n) rats immunized with rrMOG 1–125 in CFA, the experiments had to be terminated at day 15 p.i. We followed additional rats coimmunized with rrMOG 1–125 and p17 dimer in CFA at the same site up to 40 days. These rats were completely protected from EAE (Table IV, group 2). Histopathology of PBS, p45, and p17, and rrMOG 1–125 in CFA-coimmunized rats indicated absence of lesions in the CNS of p17-treated rats. In contrast, inflammation and widespread inflammation were present in rats treated with p45 or PBS (Fig. 4, *a–c*). On day 14 p.i., LEW.1N (RT1^n) immunized with rrMOG 1–125 in CFA and coimmunized with p45 or PBS alone showed inflammation (inflammatory index in p45 group ($n = 4$) 0.9 and in PBS group ($n = 4$) 0.79) and large demyelinating lesions. In contrast, LEW.1N (RT1^n) rats coimmunized with rrMOG 1–125 in CFA

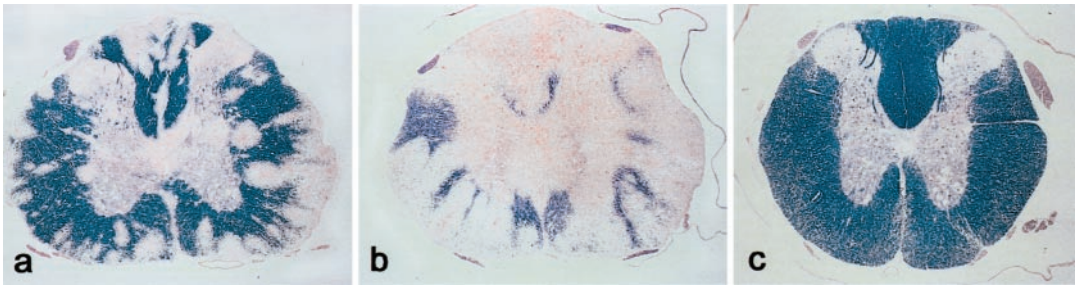

FIGURE 4. Histopathology of LEW.1N (RT1^n) rats immunized with rrMOG 1–125 and coimmunized with either PBS (*a*), p45 (*b*), or p17 (*c*) on day 14 p.i. is depicted. One representative example of four histopathologically analyzed rats per group is shown. Only coimmunization with p17, but not p45 or PBS of MOG 1–125-immunized LEW.1N (RT1^n) rats resulted in the prevention of lesion formation, as indicated by Luxol fast blue myelin staining.

together with p17 did not have inflammatory or demyelinated lesions in the CNS ($n = 4$). LEW.1N ($RT1^n$) rats coimmunized with rrMOG 1–125 in CFA together with p17 and perfused on day 19 p.i. did not show lesions either ($n = 4$; data not shown).

Third, we assessed the effect of treatment with p45 or p17 after onset of EAE. Therefore, we treated LEW.1N ($RT1^n$) rats that had been actively induced with rrMOG 1–125 in IFA on day 11 p.i. with a single dose of either p45 in IFA ($n = 10$) or p17 ($n = 17$) in IFA. Treatment with p17 resulted in strong amelioration of EAE compared with the p45-treated group ($p = 0.002$, sum score, *t* test) (Table IV, group 3).

Immune responses in EAE

We assessed the numbers of rrMOG 1–125-, MOG 91–108-, p45-, and p17-specific IFN-γ-secreting cells in the different groups immunized with rrMOG 1–125 and peptides in CFA (Fig. 5*a*). COP-1 is a synthetic copolymer composed of tyrosine (Y), glutamic acid (E), alanine (A), and lysine (K) with an average length of 40–100 aa. COP-1 mimics the physicochemical properties of MBP and is used for the immunomodulatory treatment of MS (22). One suggested mechanism of action of COP-1 is blockade of presentation of autoantigen on MHC class II molecules; the capability of COP-1 to inhibit rrMOG 1–125-induced immune responses was assessed as well (Fig. 5*a*).

Coimmunization of rrMOG 1–125 together with p17 led to strong decrease of the T cell response in the form of IFN-γ-secreting cells toward rrMOG 1–125 as compared with coimmunization with p45. Coimmunization of rrMOG 1–125 together with COP-1 led to an intermediate reduction of the number of rrMOG 1–125-specific IFN-γ-secreting cells. There were high numbers of p17-reactive IFN-γ-secreting cells in rats that had been coimmunized with rrMOG 1–125 together with p17. Peptide p45-induced IFN-γ secretion was not detectable after coimmunization of rrMOG 1–125 together with p45. There were COP-1-reactive IFN-γ-secreting cells after coimmunization of rrMOG 1–125 together with COP-1. IFN-γ-secreting cells against MOG 91–108 were not detected in peripheral lymphoid tissue in any of the three different analyzed groups (each group, $n = 5$).

Next, we assessed the Ab response level against rrMOG 1–125 in rats coimmunized with rrMOG 1–125 together with p45, p17, or COP-1 in CFA (each group, $n = 5$). Only coimmunization with p17 led to a significant decrease of the Ab titers ($p < 0.05$) (data not shown).

Immune responses of rats immunized with rrMOG 1–125 in IFA and treated on day 11 p.i. with p17 or p45 in IFA were analyzed from inguinal lymph nodes ($n = 5$ each). The application of p17 or p45 in IFA led to the nearly complete absence of T cell responses to p17. rrMOG 1–125-specific T cell responses were drastically reduced in the p17-treated LEW.1N ($RT1^n$) rats (Fig. 5*b*).

Subsequently, by real-time quantitative PCR, we assessed the expression of IL-4 and IL-10 in MNC from lymph nodes that had been restimulated in vitro for 48 h with either no Ag, rrMOG 1–125, or p17 in rrMOG 1–125-immunized, p17-immunized or p17- and rrMOG 1-125-coimmunized MOG-coimmunized LEW.1N ($RT1^n$) rats ($n = 4$ each) (Fig. 5*c*). In contrast to rrMOG 1–125-immunized rats, p17-immunized and rrMOG 1–125- and p17-coimmunized rats showed a significant up-regulation of IL-4 (restimulation with p17: rats immunized with rrMOG 1–125 compared with rats immunized with p17, $p = 0.004$; rats immunized with rrMOG 1–125 compared with rats immunized with rrMOG 1–125, and $p = 0.067$) and IL-10 (restimulation with p17: rats immunized with rrMOG 1–125 compared with rats immunized with p17, $p < 0.01$; rats immunized with rrMOG 1–125 compared with rats immunized with rrMOG 1–125 and p17, $p = 0.007$) after restimulation with p17, but not with rrMOG 1–125 (NS, *t* test).

Transfer of p45- and p17-specific T cells

Next, we assessed the potential transferability of the protective effect of p17-exposed cells. Therefore, we immunized LEW.1N ($RT1^n$) rats with p17 ($n = 9$) or p45 ($n = 9$) in CFA, and subsequently transferred 5×10^7 spleen cells to naive LEW.1N ($RT1^n$) rats that we subsequently induced for EAE with rrMOG 1–125 in IFA. Transfer of neither p45- nor p17-reactive spleen cells resulted in significant amelioration of EAE (NS, sum score, *t* test) (data not shown).

Attenuation of MBP-EAE in LEW ($RT1^l$) rats by p79

In synergy with studies for $RT1.D^n$, we also created a binding pattern for $RT1.D^l$ of LEW ($RT1^l$) rats and subsequently defined single peptides, and performed binding studies for strong or weak binding of acetylated nonapeptide amides (Tables V and VI). p79 (Ac-FWYIAIQDE-NH_2) bound with an IC_{50} of 57 nM to $RT1.D^l$, but not $RT1.B^l$ molecules ($IC_{50} > 100$ μM) nor to $RT1.D^n$ ($IC_{50} > 100$ μM) and $RT1.B^n$ ($IC_{50} > 100$ μM) molecules (Table III). The T cell response in LEW ($RT1^l$) rat EAE induced with MBP 85–99 is $RT1.D^l$ restricted (23). In contrast, EAE induced in LEW ($RT1^l$) rats with MBP 63–88 is $RT1.B^l$ restricted (23). To evaluate the isotype- and allele-specific amelioration of EAE, we coimmunized groups of LEW ($RT1^l$) rats with either MBP 85–99 alone or together with p79 (Table IV, group 4) and MBP 63–88 or MBP 63–88 together with p79 (Table IV, group 5) (each group, $n = 5$) (Table IV, group 5). Only in LEW ($RT1^l$) rats immunized with MBP 85–99 significant amelioration of EAE by coadministration of p79 could be observed ($p = 0.007$, sum score, *t* test) (Table IV, group 4). Additionally, we tested the isotype and allele specificity of p17 ($RT1.D^n$ binder) in LEW ($RT1^l$) rats. Neither treatment of p17 of LEW ($RT1^l$) rats immunized with MBP 85–99 ($RT1.D^l$ restricted) (Table IV, group 6) nor MBP 63–88 ($RT1.B^l$ restricted) (Table IV, group 7) ameliorated EAE (each group, $n = 5$). Therefore, we conclude that disease suppression with p17 or p79 in MOG-induced EAE in LEW.1N rats and MBP-induced EAE in LEW rats was MHC class II isotype and allele specific.

Allogeneic heart transplantation studies

To exclude a general immunosuppressive effect of the functional nonapeptide high affinity ligands, LEW ($RT1^l$) rats were transplanted with hearts from WF ($RT1^u$) rats. LEW ($RT1^l$) rats carrying heterotopic WF hearts were treated with p79 ($n = 4$), a syngeneic control peptide ($n = 7$), or PBS ($n = 6$). There was no significant delay of heart function in LEW ($RT1^l$) rats after treatment with p79 (6.5 ± 0.58 days) as compared with control peptide (7 ± 0.5 days) or PBS (6.5 ± 0.5 days). These results argue against general immunosuppressive properties of p79 in LEW ($RT1^l$) rats.

In vitro blocking of IFN-γ reduction of MOG-specific T cells by p17

Finally, we tested whether MOG-specific T cells eluted from the CNS could be blocked by p17. T cells were eluted from the CNS of diseased LEW.1N ($RT1^n$) rats and incubated with irradiated thymocytes in the presence or absence of MOG 91–108 alone or MOG 91–108 and p17 in parallel. Addition of 10 or 50 μg of p17 led to a significant reduction of the numbers of MOG 91–108-specific IFN-γ-secreting cells, as shown in Fig. 6 ($n = 6$ each; MOG 91–108 vs MOG 91–108 plus p17, 10 μg/ml, $p < 0.05$; MOG 91–108 vs MOG 91–108 plus p17, 50 μg/ml, $p < 0.01$).

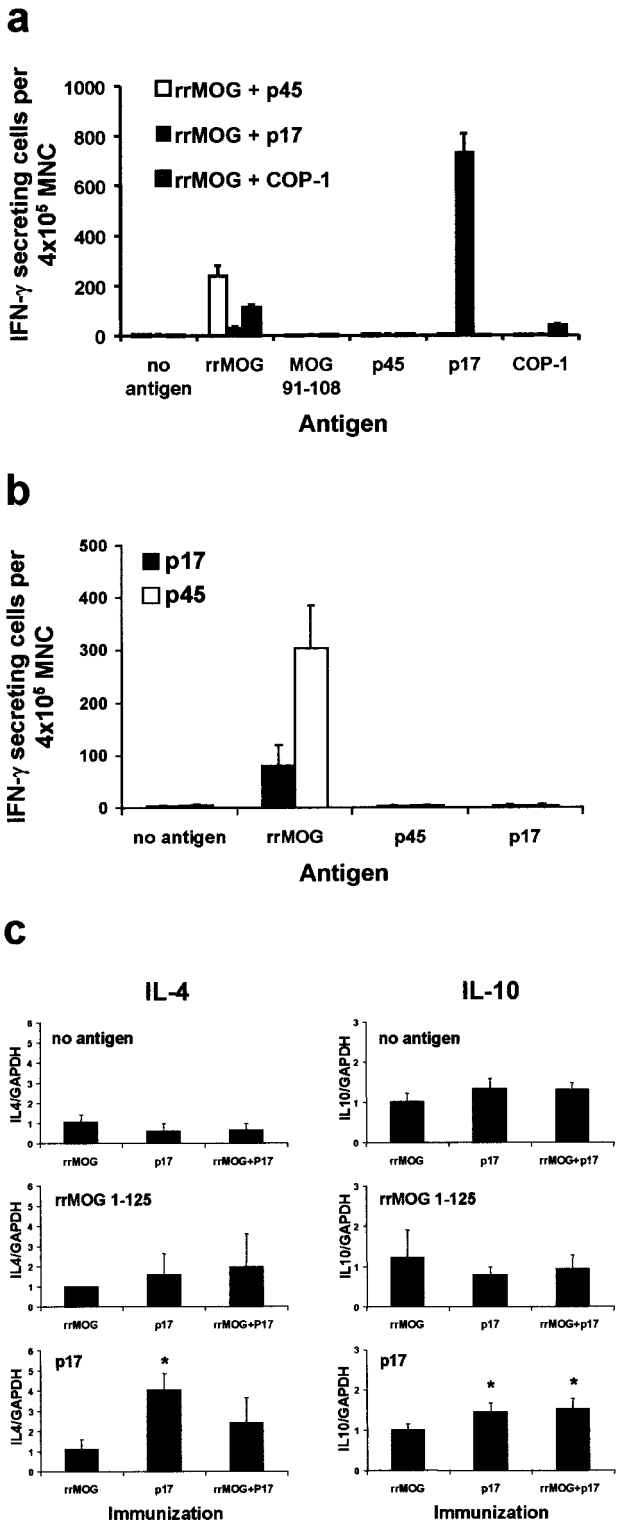

FIGURE 5. Influence of p17 and p45 on autoimmune T and B cell responses. *a*, Numbers of IFN-γ-secreting cells in LEW.1N (RT1^n) rats coimmunized with either rrMOG 1–125 and p45, rrMOG 1–125 and p17, or rrMOG 1–125 and COP-1 in CFA. *b*, Numbers of IFN-γ-secreting cells in LEW.1N (RT1^n) rats with rrMOG 1–125-induced EAE treated with either p17 or p45 in IFA at day 11 p.i. *c*, MNC from LEW.1N (RT1^n) rats immunized with either rrMOG 1–125 alone, p17 alone, or rrMOG 1–125 and p17 together were cultured for 48 h with either no Ag (*upper panel*), rrMOG 1–125 (*middle panel*), or p17 (*lower panel*). Subsequently, mRNA expression for IL-4 and IL-10 was detected by real-time quantitative PCR. There were increased mRNA values after restimulation with p17 for IL-4 in p17-immunized LEW.1N (RT1^n) rats ($p = 0.004$), and a trend for rats coimmunized with p17 and rrMOG 1–125 ($p = 0.067$). A similar increase was observed after restimulation with p17 for IL-10 in p17-immunized ($p = 0.01$) and rrMOG 1–125- and p17-coimmunized rats ($p = 0.007$). *, Indicates statistical significance.

Table V. *Influence of defined amino acid residues on competitive binding to RT1.D^l*

Sequence Position	Favorable	Unfavorable
1	F H K R W Y	A I L M V
2	A V W Y	H I L
3	M V	D E G N
4	I L M N	A E G H
5	A I L	D E G N
6	I L N V	D E H K R W
7	A G Q	F W Y
8	A D E G P	I W Y
9	D E	F L W Y

Discussion

We have demonstrated that MOG 91–108- and MOG 1–125-induced EAE in LEW.1N (RT1^n) rats can be prevented or ameliorated by a synthetic high affinity RT1.D^n ligand, p17 (Ac-FWFLDNAPL-NH_2), which is unrelated to MOG. In synergy, MBP 85–99 (RT1.D^l restricted)-, but not MBP 63–88 (RT1.B^l restricted)-induced EAE in LEW (RT1^l) rats could be ameliorated by the MBP-nonrelated RT1.D^l high affinity ligand p79 (Ac-FWYIAIQDE-NH_2), but not p17, demonstrating the isotype and allele specificity of the intervention. A database search did not reveal any sequence similarity to self molecules for these ligands (data not shown). The newly designed high affinity ligands p17 as well as p2, p3, and p11 were highly immunogenic if injected in CFA in LEW.1N (RT1^n) rats. The presentation of these acetylated peptide amides was limited to RT1.D^n because only coculture with mAb OX-17 (anti-RT1.D), but not mAb OX-6 (anti-RT1.B) resulted in blocking of T cell responses.

Although these synthetic peptides are expected to be nonself to the T cell repertoire of the LEW.1N (RT1^n) rat, it was not excluded that the peptides could mimic unknown self peptides with thymic deletion of the reactive T cell repertoire as a consequence (24). The presence of a T cell repertoire to each of these peptides indicates that presented self-unrelated high affinity ligands could be important for the control of autoimmunity in general. First, by competition for presentation at the immunological synapse they can prevent the display of autoantigen-derived peptides to T cells (25–27); second, they can bias the T cell repertoire toward high avidity T cells, filling up empty space in the repertoire, and suppress low avidity T cell response that could be associated with autoimmunity (28). Third, they can induce T cells with a regulatory phenotype expressing IL-4 and IL-10.

Potentially, the induction of immune responses against high affinity ligands could result in autoimmunity by molecular mimicry at least in conjunction with a certain T cell repertoire (29). Our study underscores such a scenario, because we demonstrate that in the context of an inflammatory stimulus (CFA) all investigated high affinity MHC class II ligands raised immune responses. Nevertheless, this possibility is very low due to the extremely high number of nonself sequences the body is encountering in its life in the context of host defense mechanisms that do not result in autoimmunity.

This is the first report on the peptide-binding specificities of RT1.D molecules. We gathered information on the RT1.D^n- and

Table VI. *IC_{50} values of acetylated nonapeptide amides for binding to RT1.D^l and prediction of binding properties*[a]

Peptide No.	Sequence									Prediction[b]	IC_{50} (μM)
p79	F	W	Y	I	A	I	Q	D	E	+	0.057
p68	W	W	W	M	L	I	Q	G	E	+	0.06
p67	F	Y	M	M	I	I	P	A	E	+	0.08
p80	W	A	F	I	I	I	Q	E	E	+	0.31
p78	R	Y	W	N	L	V	P	P	E	+	0.39
p71	K	Y	V	I	I	L	P	P	D	+	0.55
p75	Y	W	F	M	I	N	G	E	D	+	1.0
p74	W	Y	Y	L	A	N	A	E	D	+	1.4
p63	Y	Y	W	I	L	N	P	P	D	+	2.6
p73	F	V	W	L	A	N	A	D	D	+	6.3
p90	M	I	N	G	D	W	F	I	F	−	8.0
p64	H	W	Y	I	A	V	Q	D	D	+	13
p89	A	H	G	H	E	R	W	Y	L	−	35
p66	R	V	V	L	I	V	G	A	E	+	55
p62	W	V	M	N	I	N	G	G	D	+	65
p87	L	I	D	G	N	D	W	I	W	−	82
p61	F	A	V	N	A	N	A	A	D	+	>100
p65	K	A	F	L	A	V	A	E	D	+	>100
p69	Y	A	Y	N	L	I	A	G	E	+	>100
p70	H	V	F	N	A	L	G	P	E	+	>100
p72	R	A	M	I	L	L	Q	D	D	+	>100
p76	H	A	V	M	I	V	G	A	E	+	>100
p77	K	V	M	N	L	V	P	G	E	+	>100
p81	I	I	G	G	E	D	F	I	L	−	>100
p82	L	L	N	G	E	E	F	W	F	−	>100
p83	V	H	D	A	D	K	F	Y	W	−	>100
p84	A	I	E	A	D	R	Y	I	Y	−	>100
p85	M	L	G	H	G	W	Y	W	L	−	>100
p86	I	H	N	H	G	H	Y	Y	F	−	>100
p88	V	L	E	A	N	K	W	W	Y	−	>100

[a] All peptides are N-terminally acetylated and C-terminally amidated.

[b] The peptides marked "+" consist of randomly selected combinations of favorable amino acids, whereas the peptides marked "−" consist of randomly selected combinations of unfavorable amino acids, according to Table V.

RT1.D^l-binding specificities using combinatorial peptide libraries. Various human HLA-DR and mouse H2-E molecules have been crystallized with and without peptides in the peptide-binding groove, and structural analysis revealed binding of a nonapeptide core sequence with anchor residues at positions 1, 4, 6, and 9 (30, 31). Moreover, pocket 1 (P1) has been shown to be preferentially occupied by aromatic and aliphatic residues. We also found a preference for aromatic amino acids in position 1 of the HLA-DR-like RT1.D^n molecule. Although the approach described in this work did not allow assigning anchor positions in homology with HLA-DR and H2-E molecules, we found that positions 3 and 6 of p17 (Ac-FWFLDNAPL-NH_2) were most important for binding. A or K substitutions at these positions resulted in dramatic reduction of binding. Truncated hexapeptide variants of p17 still bound to RT1.D^n. Further truncations resulted in loss of binding. Acetylation at the N-terminal part of the peptides strongly enhanced binding.

In the past, several studies have addressed the possibility of inhibiting experimentally induced autoimmunity in rodents with Abs or peptides inhibiting the presentation of the autoantigen at the MHC level. Steinman et al. (32) have shown in SJL/J (H-2^s) mice that blockade of MHC class II presentation with a mAb can lead to prevention of EAE. Subsequently, Wraith et al. (33) have demonstrated that a peptide based on rat MBP Ac1–11 (Ac-ASQKRPSQRHG) substituted on position 4 with alanine can be used to attenuate EAE induced with MBP Ac1–11 in (PL/J × SJL)F_1 (H-2^{u+s}) mice. This approach has been followed by others (34, 35). However, if the competitor peptide is structurally closely related to the pathogenic peptide, other mechanisms than inhibiting

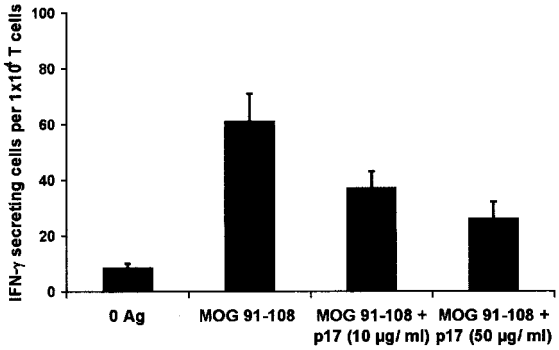

FIGURE 6. Effects of p17 on IFN-γ secretion of T cells eluted from the CNS of MOG-immunized LEW.1N (RT1^n) rats in the presence of Ag. T cells were eluted from the CNS of LEW.1N (RT1^n) rats on day 9 p.i., and subsequently restimulated with irradiated thymocytes in the presence of Ag. Subsequently, ELISPOT analysis for enumeration of IFN-γ-secreting cells was performed. Addition of p17 to MOG 91–108-restimulated T cells led to reduction of numbers of MOG 91–108-specific IFN-γ-secreting cells ($n = 6$ each; MOG 91–108 vs MOG 91–108 plus p17, 10 μg/ml, $p < 0.05$; MOG 91–108 vs MOG 91–108 plus p17, 50 μg/ml, $p < 0.01$).

presentation of autoantigen-derived peptide at the level of the MHC class II molecules play a critical role.

T cell determinants from known self or nonself proteins were used that were unrelated to the autoantigenic peptide sequence to inhibit induced autoimmune disease (27, 36). Hurtenbach et al. (27) defined a ligand (yTYTVHAAHAYTYt, small letters indicate D amino acids) selected by in vitro inhibition studies against an hen egg lysozyme peptide 8–29-specific T cell hybridoma restricted to the MHC class II allele H2-A^{g7} of the diabetes-prone NOD mouse that inhibited onset of diabetes in vivo. This ligand bound with an IC_{50} of ~1 μM to H2-A^{g7}. In such approaches, the selection of the competing determinant strictly depends on the availability of information on relevant T cell epitopes. Single amino acid modifications of the selected peptides to improve binding efficiency do not necessarily generate ligands with very high affinity. Our approach has the definitive advantage that it guarantees the generation of ligands with the highest possible affinity and reduces the risk of generating undesired altered peptide ligand effects. Additionally, acetylation and amidation further improved binding and stabilization against exopeptidases. Moreover, the small size of the peptides of 9 aa offers the important advantage that binding is restricted to a minimum of core residues and is therefore highly isotype and allele specific.

MOG-induced EAE in rats mimics many hallmarks of MS, such as CNS lesions with demyelination and axonal and neuronal loss (16–18, 21, 37, 38). This model is therefore highly relevant for investigations of disease pathogenesis and treatment strategies for MS. Interestingly, in LEW.1N (RT1^n) rats, MOG 91–108 does not induce an ex vivo detectable T cell response in peripheral lymphoid tissue, but a strong cellular response is detectable in the CNS (18). Upon coimmunization of MOG 1–125 or MOG 91–108 and p17 in CFA in LEW.1N (RT1^n) rats, disease was completely abrogated, indicating that there was prevention of intracerebral autoimmunity. We have demonstrated that p17 binds to intact MHC class II molecules on the cell surface. Our results indicate that p17 with an IC_{50} of 2 nM efficiently competes with MOG 91–108 for presentation on RT1.D^n, because MOG 91–108 has a higher IC_{50} of 30 nM.

We performed in vitro studies of T cells that had been eluted from the CNS and subsequently exposed to Ag in the presence of

irradiated thymocytes. The addition of p17 led to a reduction of the MOG 91–108-specific T cell responses. These data underscore that p17 is competing at the immunological synapse for presentation of autoantigen. In light of these results in vitro, our in vivo studies would also strongly indicate that p17 acts by competition for presentation of autoantigen on APC.

Not only is p17 capable of blocking disease upon coimmunization with either MOG 91–108 or rrMOG 1–125, rrMOG-induced EAE could also be treated by the i.p. application of p17. Moreover, application of the encephalitogen and p17 at the same time point, but at different sites of the body, led to a significant attenuation of EAE. This cannot solely be explained by competition at the level of the restrictive MHC class II molecule. Although coimmunization and treatment of EAE with p17 led to a clear reduction of IFN-γ-secreting cells in response to rrMOG 1–125, IL-4 and IL-10 measurements did not indicate a dramatic Th1/Th2 shift. Moreover, the protective effect of p17 could not be transferred. p17 as well as the other high affinity ligands tested raised a considerable IFN-γ response upon immunization. Therefore, it can be assumed that disease protection in the context of inflammation not only occurred because of competition at the MHC level, but also because of the immune response generated against p17 with presence of high avidity T cells suppressing the low avidity MOG-specific self-reactive T cell repertoire (28).

There is a multitude of mechanisms discussed in the context of the action of COP-1 (22). Recently, it has been shown that modified COP-1 variants improved clinical efficiency (39). The variants with the best clinical effect showed improved fitting to MHC class II molecules. Our study regarding the effects of p17 would underscore that the mechanism of COP-1 in MS is competition for binding at the level of MHC class II presentation. In contrast to COP-1 and its variants, p17 and related nonamer peptides have a nearly absolute MHC class II isotype and allele specificity and show improved binding. As we have demonstrated for p17 and COP-1 at equimolar concentrations, p17 and related peptides are of superior efficiency compared with COP-1 to reduce MOG-specific immune responses.

We have demonstrated that MOG-induced EAE in rats can be attenuated by highly selective isotype- and allele-specific intervention, targeting disease-associated MHC class II molecules at the immunological synapse. We did not observe undesired side effects such as anaphylactic reactions, enhanced autoimmunity, or general immunosuppression. Therefore, peptides or peptidomimetics selectively intervene with presentation of autoantigenic peptides on disease-associated MHC class II isotype allelic variants. The therapeutic potential of this strategy merits further investigations in clinical trials for treatment of autoimmune diseases of humans.

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Publikation 7

High Immunogenicity of Intracellular Myelin Oligodendrocyte Glycoprotein Epitopes[1]

Robert Weissert,[2]* Jens Kuhle,* Katrien L. de Graaf,* Wolfgang Wienhold,* Martin M. Herrmann,* Claudia Müller,† Thomas G. Forsthuber,‡ Karl-Heinz Wiesmüller,§ and Arthur Melms*

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the CNS with associated axonal loss. There is strong evidence for an autoimmune pathogenesis driven by myelin-specific T cells. Myelin oligodendrocyte glycoprotein (MOG) induces a type of experimental autoimmune encephalomyelitis in animals which is very MS-like since there are demyelinating CNS lesions and axonal loss. This underscores the potential role of MOG in MS pathogenesis. We performed a T cell reactivity pattern analysis of MS patients at the onset of relapse or progression of neurological deficits and controls that were stratified for the genetic risk factor HLA-DRB1*1501. For the first time, we show that there is an HLA-DR-restricted promiscuous dominant epitope for $CD4^+$ T cells within the transmembrane/intracellular part of MOG comprising aa 146–154 (FLCLQYRLR). Surprisingly, controls had broader T cell reactivity patterns toward MOG peptides compared with MS patients, and the transmembrane and intracellular parts of MOG were much more immunogenic compared with the extracellular part. Measurements of in vitro binding affinities revealed that HLA-DRB1*1501 molecules bound MOG 146–154 with intermediate and HLA-DRB1*0401 molecules with weak affinities. The binding of MOG 146–154 was comparable or better than myelin basic protein 85–99, which is the dominant myelin basic protein epitope in context with HLA-DRB1*1501 molecules in MS patients. This is the first study in which the data underscore the need to investigate the pathogenic or regulatory role of the transmembrane and intracellular part of MOG for MS in more detail. ***The Journal of Immunology,*** **2002, 169: 548–556.**

Multiple sclerosis (MS)[3] is an inflammatory and demyelinating disease of the CNS with major socioeconomic impact (1). Besides environmental stimuli, genetic factors determine disease outbreak. There is an increased risk for patients being HLA-DRB1*1501, DRB5*0101, and DQB1*0602, which are contained in the Dw2 haplotype to develop MS, and HLA-DRB1*1501 is associated with an earlier age of disease manifestation (2). Myelin-specific autoreactive T cells pass the blood brain barrier and initiate an autoimmune attack against myelin sheaths in the CNS (3). Depending on the degree of the myelin sheath-specific autoimmune attack, axons can also be harmed, leading to irreversible axonal loss and long-term global brain atrophy (4). Several myelin proteins are thought to be targets of tissue damage in MS (5). Much attention has been given to myelin-basic protein (MBP) and proteolipid protein. More recently, myelin oligodendrocyte glycoprotein (MOG) has gained considerable attention with regard to the autoimmune attack in MS (6). This is due to the high histopathological similarity between MOG-induced animal models and biopsies from MS patients, both showing large demyelinating lesions and axonal loss (7, 8). MOG only composes ~0.01% of the protein content of the myelin sheath and is exposed on its outer surface (6). It has an extracellular part including aa 1–122 with an Ig-like domain, a transmembrane part, and an intracellular part comprising aa 123–218 (Fig. 7; Refs. 9–11). Up to now, T cell responses against the extracellular part of MOG have been mainly looked at. Using the ELISPOT methodology, Wallström et al. (12) demonstrated increased IFN-γ-secreting cells in HLA-DR2(15) positive MS patients compared with controls toward several MOG peptides among which MOG 63–87 was immunodominant. Others investigated T cell responses to MOG of non-HLA-stratified MS patients and controls indicating increased T cell reactivity to MOG in MS patients (13–15). Also, MOG-specific B cell responses are up-regulated in MS, and MOG-specific Abs contribute to lesion formation (16–17). Because MOG can be considered an important autoantigen in MS and a potential target for therapy, we investigated T cell responses by ELISPOT assay for IFN-γ-secreting cells to peptides of the complete human MOG 1–218 sequence. We used the ELISPOT assay to detect IFN-γ-secreting cells, because this is more sensitive than proliferation as assessed by [^{3}H]TdR-uptake, and adds a functional outread. We demonstrate a previously unknown dominant T cell epitope that is recognized in context of HLA-DR molecules by $CD4^+$ T cells within the transmembrane and intracellular sequence 146–154 of MOG in MS patients and controls.

Materials and Methods

Subjects

MS patients and controls were stratified for HLA-DRB1*1501. This resulted in four groups with 11 MS patients and 10 controls being HLA-DRB1*1501 positive, and 7 MS patients and 10 controls being HLA-DRB1*1501 negative. A total of 13 patients had a relapsing-remitting MS disease course with an average disease duration at a sample collection of 1 year and 6 mo (mean expanded disability status scale (EDSS) 2.0). Four

Departments of *Neurology and †Transplantationimmunology and Immunehematology, University of Tübingen, Tübingen, Germany; ‡Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106; and §EMC Microcollections, Tübingen, Germany

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[2] Address correspondence and reprint requests to Dr. Robert Weissert, Department of Neurology, University of Tübingen, Hoppe-Seyler-Strasse 3, 72076 Tübingen, Germany. E-mail address: robert.weissert@uni-tuebingen.de

[3] Abbreviations used in this paper: MS, multiple sclerosis; CM, complete medium; EAE, experimental autoimmune encephalomyelitis; EDSS, expanded disability status scale; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; rhMOG, recombinant human MOG.

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patients had a secondary progressive disease course at sample collection with an average disease duration of 8 years (mean EDSS 5.0), and one patient had a primary progressive disease course with a duration of 7 years at sample collection (EDSS 6.5). Sixteen of 18 patients had not been treated at all or had not received any immunosuppressive or immunomodulatory treatment for at least 1 year before sample collection. Two of 18 patients had not received immunosuppressive or immunomodulatory treatment for 1 mo before sample collection. All patients were admitted to Department of Neurology of the University of Tubingen (Tubingen, Germany) due to acute relapse (relapsing remitting disease course) or fast worsening of neurological deficits (primary or secondary progressive disease course). The mean age of MS patients was 34 ± 9 years and of controls 38 ± 11 years, with a male to female ratio of 50:50% in MS patients and 55:45% in controls. The age distribution and female:male ratio was equal in the HLA-DRB1*1501 positive and negative groups. The studies had been approved by the ethical review board of the University of Tübingen.

HLA-typing

Genomic DNA for HLA-genotyping was prepared by the QIAampBlood kit (Qiagen, Hilden, Germany). Low resolution pregenotyping was performed for the 116 major HLA-DRB1, -DRB3, -DRB4, -DRB5, and 29 HLA-DQB alleles, and HLA-DR or HLA-DQ subtyping was done specifically for the pretyped HLA-DRB1/DQB1 alleles by group-specific amplification and subsequent direct sequencing in patients and controls.

Cloning and bacterial expression of recombinant human (rh)MOG 1–125

The cDNA of rhMOG was obtained by reverse transcription of total RNA from a human glioma cell line. RNA was prepared with TRIzol reagent (Life Technologies, Gaithersburg, MD) and first strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and the gene-specific primer R785. The amplified PCR product that contains the whole coding sequence of MOG mRNA was separated by agarose gel electrophoresis and purified with a QIAEXII-kit (Qiagen). The cDNA fragment was cloned into Bluescript II KS^+ vector (Stratagene, La Jolla, CA). The DNA sequence encoding the extracellular domain of the mature protein (including four N-terminal amino acids of the transmembrane domain) was PCR-amplified and subcloned in pQ60 (Qiagen). The *Escherichia coli*-expressed His-tagged fusion protein (rhMOG 1–125) was purified under denaturing conditions by metal chelate affinity chromatography on Ni-NTA agarose columns (Qiagen) according to the manufacturer's guidelines.

Synthesis and analysis of peptides

The 16 aa-long peptides (Table I) and the N-acetylated C-amidated 9 aa-long peptides (Table II) were prepared by solid phase peptide synthesis using F-moc/tBu chemistry. The peptides were purified by preparative HPLC (Abimed, Langenfeld, Germany). The identity of the purified peptides was confirmed by electrospray mass spectrometry. The purity of peptides was >95% as determined by analytical HPLC (Abimed). MBP 85–99 (ENPVVHFFKNIVTPR) and influenza A peptide (YRNLVWFIKKNTRYP) (18) were synthesized and purified in the same way. The peptides were used at a concentration of 10 μg/ml in all experiments. This concentration had given the highest number of spots in ELISPOT analyses in pilot experiments (data not shown).

Mitogen

PHA was purchased from Sigma-Aldrich (Deisenhofen, Germany) and used in a concentration of 3 μg/ml in all experiments. This concentration had given optimal results in preceding experiments.

Purification of PBMC

PBMCs were isolated from heparinized blood samples on Lymphoprep density gradients (Nyegaard, Oslo, Norway; 200 × *g*, 25 min, room temperature). Cells were frozen at a density of 5×10^6 cells/ml in freezing medium containing 40% FCS Gold (PAA, Linz, Austria), 10% DMSO (Serva, München, Germany), and 50% complete medium (CM) consisting of RPMI 1640 (Life Technology, Eggenstein, Germany) supplemented with 2 mM glutamine (Life Technology), 100 U/ml of penicillin, 100 μg/ml of streptomycin (Biochrom, Berlin, Germany), and 3% heat-inactivated human AB serum in liquid nitrogen. The cells were recovered by thawing in a 37°C water bath until the cells reached the melting point and then by adding 1 ml CM per 5×10^6 cells three times every 5 min at room temperature. Thereafter, the cells were washed three times in CM and resuspended at a density of 10^6/ml in CM. The viability was 90–95%.

Table I. *List of peptides spanning the complete MOG sequence*

Peptide	Sequence
MOG 1–16	GQFRVIGPRHPIRALV
MOG 5–20	VIGPRHPIRALVGDEV
MOG 9–24	RHPIRALVGDEVELPC
MOG 13–28	RALVGDEVELPCRISP
MOG 17–32	GDEVELPCRISPGKNA
MOG 21–36	ELPCRISPGKNATGME
MOG 25–40	RISPGKNATGMEVGWY
MOG 29–44	GKNATGMEVGWYRPPF
MOG 33–48	TGMEVGWYRPPFSRVV
MOG 37–52	VGWYRPPFSRVVHLYR
MOG 41–56	RPPFSRVVHLYRNGKD
MOG 45–60	SRVVHLYRNGKDQDGD
MOG 49–64	HLYRNGKDQDGDQAPE
MOG 53–68	NGKDQDGDQAPEYRGR
MOG 57–72	QDGDQAPEYRGRTELL
MOG 61–76	QAPEYRGRTELLKDAI
MOG 65–80	YRGRTELLKDAIGEGK
MOG 69–84	TELLKDAIGEGKVTLR
MOG 73–88	KDAIGEGKVTLRIRNV
MOG 77–92	GEGKVTLRIRNVRFSD
MOG 81–96	VTLRIRNVRFSDEGGF
MOG 85–100	IRNVRFSDEGGFTCFF
MOG 89–104	RFSDEGGFTCFFRDHS
MOG 93–108	EGGFTCFFRDHSYQEE
MOG 97–112	TCFFRDHSYQEEAAME
MOG 101–116	RDHSYQEEAAMELKVE
MOG 105–120	YQEEAAMELKVEDPFY
MOG 109–124	AAMELKVEDPFYWVSP
MOG 113–128	LKVEDPFYWVSPGVLV
MOG 117–132	DPFYWVSPGVLVLLAV
MOG 121–136	WVSPGVLVLLAVLPVL
MOG 125–140	GVLVLLAVLPVLLLQI
MOG 129–144	LLAVLPVLLLQITVGL
MOG 133–148	LPVLLLQITVGLVFLC
MOG 137–152	LLQITVGLVFLCLQYR
MOG 141–156	TVGLVFLCLQYRLRGK
MOG 145–160	VFLCLQYRLRGKLRAE
MOG 149–164	LQYRLRGKLRAEIENL
MOG 153–168	LRGKLRAEIENLHRTF
MOG 157–172	LRAEIENLHRTFDPHF
MOG 161–176	IENLHRTFDPHFLRVP
MOG 165–180	HRTFDPHFLRVPCWKI
MOG 169–184	DPHFLRVPCWKITLFV
MOG 173–188	LRVPCWKITLFVIVPV
MOG 177–192	CWKITLFVIVPVLGPL
MOG 181–196	TLFVIVPVLGPLVALI
MOG 185–200	IVPVLGPLVALIICYN
MOG 189–204	LGPLVALIICYNWLHR
MOG 193–208	VALIICYNWLHRRLAG
MOG 197–212	ICYNWLHRRLAGQFLE
MOG 201–216	WLHRRLAGQFLEELRN
MOG 205–218	RLAGQFLEELRNPF

ELISPOT analysis for IFN-γ-secreting cells and restriction analysis

Ninety six-well nitrocellulose plates (Millipore, Molsheim, France) were coated with 10 μg/ml capture mAb 1-D1K (Mabtech, Stockholm, Sweden) overnight at 4°C. After washing, the membrane was blocked with culture medium containing 10% FCS (FCS Gold, PAA) for 1 h. A total of 2×10^5 PBMCs/well were cultured for 40 h in 37°C, 5% CO_2. For each Ag or mitogen, triplicates were used. Each plate contained a positive and negative control. All peptides and mitogens were tested for one patient or control at the same time point with exactly the same procedures. After 40 h, the cells were discarded and the membranes were thoroughly washed by immersing the plates six times in PBS. To visualize areas of the membrane that had bound secreted IFN-γ, biotinylated detector mAb 7-6B-1 (1 μg/ml, Mabtech) was added for 3 h and staining performed with avidin-biotin peroxidase complex (Vectastatin Elite ABC kit; Vector Laboratories, Burlingame, CA) and chromogen solution containing carbazole (Sigma-Aldrich). Areas of the membrane where a specific color reaction had occurred appeared as dark brown-red spots and were both counted by an automated

Table II. *List of truncated N-acetylated C-amidated MOG peptides of sequence MOG 142–160*

Peptide	Sequence
MOG 142–150	Ac-VGLVFLCLQ-NH_2
MOG 143–151	Ac-GLVFLCLQY-NH_2
MOG 144–152	Ac-LVFLCLQYR-NH_2
MOG 144–152 V 148	Ac-LVFLVLQYR-NH_2
MOG 145–153	Ac-VFLCLQYRL-NH_2
MOG 146–154	Ac-FLCLQYRLR-NH_2
MOG 147–155	Ac-LCLQYRLRG-NH_2
MOG 148–156	Ac-CLQYRLRGK-NH_2
MOG 149–157	Ac-LQYRLRGKL-NH_2
MOG 150–158	Ac-QYRLRGKLR-NH_2
MOG 151–159	Ac-YRLRGKLRA-NH_2
MOG 152–160	Ac-RLRGKLRAE-NH_2

ELISPOT counter (Autoimmun-Diagnostika, Albstadt, Germany) and manually cross-checked. The average number of spots in triplicates secreted after exposure with Ag or mitogen were expressed as numbers of IFN-γ-secreting cells per 2×10^5 cells added initially to the wells. For the restriction analysis, the HLA typing Abs Genox with the specificity anti-HLA-DQ and/or Tü 36 with the specificity anti-HLA-DR were added at a concentration of 10 μg/ml to the cultures. These concentrations of Abs had given optimal results in pilot experiments (data not shown).

Enrichment of IFN-γ-secreting cells and FACS analyses

Enrichment of cells secreting IFN-γ after contact with Ag was performed with magnetic cell separation as described by the manufacturer (IFN-γ secretion assay; Miltenyi Biotec, Bergisch Gladbach, Germany). PBMC from MS patients and controls were incubated with no Ag, MOG 146–154 (10 μg/ml), or as a positive control staphylococcal enterotoxin B (Sigma-Aldrich, 10 μg/ml) for 12 h at a concentration of 5×10^6 cells/ml at 37°C, 5% CO_2. Thereafter, cells were exposed to a bivalent Ab to IFN-γ and leukocyte surface Ag (Miltenyi Biotec) at 4°C resulting in an activity matrix for IFN-γ on the cell surface. Subsequently, cells were reinduced to secrete IFN-γ at 37°C, 5% CO_2. Secreted IFN-γ is captured by the activity matrix on the cell surface. Next, cells were incubated with a PE-labeled Ab to IFN-γ (Miltenyi Biotec). Finally, cells with bound IFN-γ on their cell surface were separated with a PE-specific microbead (Miltenyi Biotec) in a magnetic field. Cells were analyzed for expression of IFN-γ (mouse PE-labeled anti-human IFN-γ; Miltenyi Biotec) and CD4 (mouse FITC-labeled anti-human CD4; BD Biosciences, Lincoln Park, NJ) and/or CD8 (mouse FITC-labeled anti-human CD8) expression following standard procedures by FACS (BD Biosciences).

Peptide binding assay

Relative affinities of MOG peptides for purified HLA-DRB1*1501 and HLA-DRB1*0401 molecules were measured by an inhibition ELISA based on a dissociation-enhanced lanthanide fluoroimmunoassay (Wallac, Turku, Finland). In the inhibition ELISA, HLA-DR (50 nM) molecules were incubated with fixed amounts of respective tracer peptides (10–50 nM) in the presence of a range of dilutions of the unlabeled MOG-peptides (10-fold dilutions between 1 nM and 100 μM). The binding buffer consisted of a carbonate buffer titrated to pH 5 containing 2 mM EDTA, 0.01% azide, 0.1 mM PMSF, and 0.1% Nonidet P-40 (Boehringer Mannheim, Indianapolis, IN). After an incubation of 48 h at 37°C, the peptide-MHC complexes were transferred to Ab-coated (L243) ELISA plates (FluoroNunc; Nunc, Roskilde, Denmark) to remove the excess of nonbound peptides. Europium-labeled streptavidin (Wallac) was added to the plates and incubated for 1 h at room temperature. Finally, the plates were treated with an enhancement solution (Wallac), which releases chelated europium from streptavidin and forms a highly fluorescent solution that can be measured by using a dissociation-enhanced lanthanide fluoroimmunoassay fluorometer (Wallac). The peptide concentration yielding 50% inhibition of binding of the tracer peptide (IC_{50}) was determined by plotting the percentage of inhibition vs the concentration of added MOG peptide. Peptides were tested in two to three independent experiments.

Statistical analysis

Repeated measures ANOVA on signed ranks were used with the Dunnett's test (Sigma Stat; Jandel Scientific, San Rafael, CA). The Friedman repeated measures analysis of variance on ranks (repeated measures ANOVA on signed ranks) is a parametric test that compares effects of a series of different experimental treatments on a single group. Each subject's responses are ranked from smallest to largest without regard to other subjects, then the rank sums for the subjects are compared. Dunnett's test is the analog of the Student-Newman-Keuls test for the case of multiple comparisons against a single control group. It is conducted similarly to the Bonferroni *t* test, but with a more sophisticated mathematical model of the way the error accumulates to derive the associated table of critical values for hypothesis testing. This test is less conservative than the Bonferroni test, and is only available for multiple comparisons vs a control. This analysis was performed independently for each of the four groups (DRB1*1501 positive MS patients, DRB1*1501 positive controls, DRB1*1501 negative MS patients, DRB1*1501 negative controls). As controls, the individual background reactivities were used (T cell responses without Ag).

Results

T cell repertoire analysis to the extracellular part of MOG

We stimulated PBMCs from MS patients and controls with 52 overlapping peptides covering the complete human MOG sequence (Table I) or with the extracellular rhMOG 1–125 (mature peptide, aa 1–125) and enumerated IFN-γ-secreting cells with the ELISPOT assay (Figs. 1 and 2). The T cell responses as assessed by IFN-γ secretion toward peptides of the extracellular part of MOG were weak and heterogeneous. HLA-DRB1*1501 positive controls showed responses to peptide MOG 81–96 ($p < 0.05$) (Fig. 2), while MS patients and HLA-DRB1*1501 negative controls reacted weakly (NS). Some controls reacted with MOG 73–88 (NS), but not MS patients (NS). Only a few individuals had IFN-γ-producing cells that reacted with rhMOG 1–125 (NS).

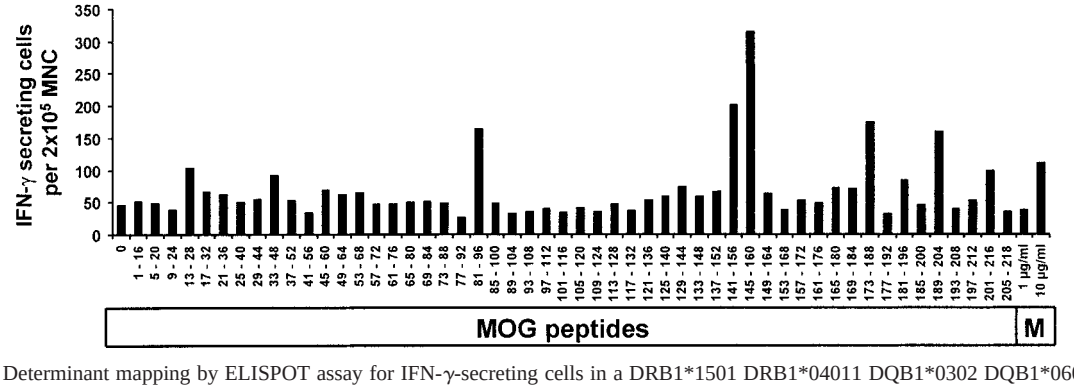

FIGURE 1. Determinant mapping by ELISPOT assay for IFN-γ-secreting cells in a DRB1*1501 DRB1*04011 DQB1*0302 DQB1*0602 MS patient. IFN-γ secreting mononuclear cells from peripheral blood reactive with 16 aa-long peptides covering the complete MOG sequence and toward rhMOG 1–125 are indicated. The number of spots indicating cells having secreted IFN-γ after antigenic stimulus was enumerated with an automated analysis system. The procedures were performed as described in *Materials and Methods*. M indicates rhMOG 1–125.

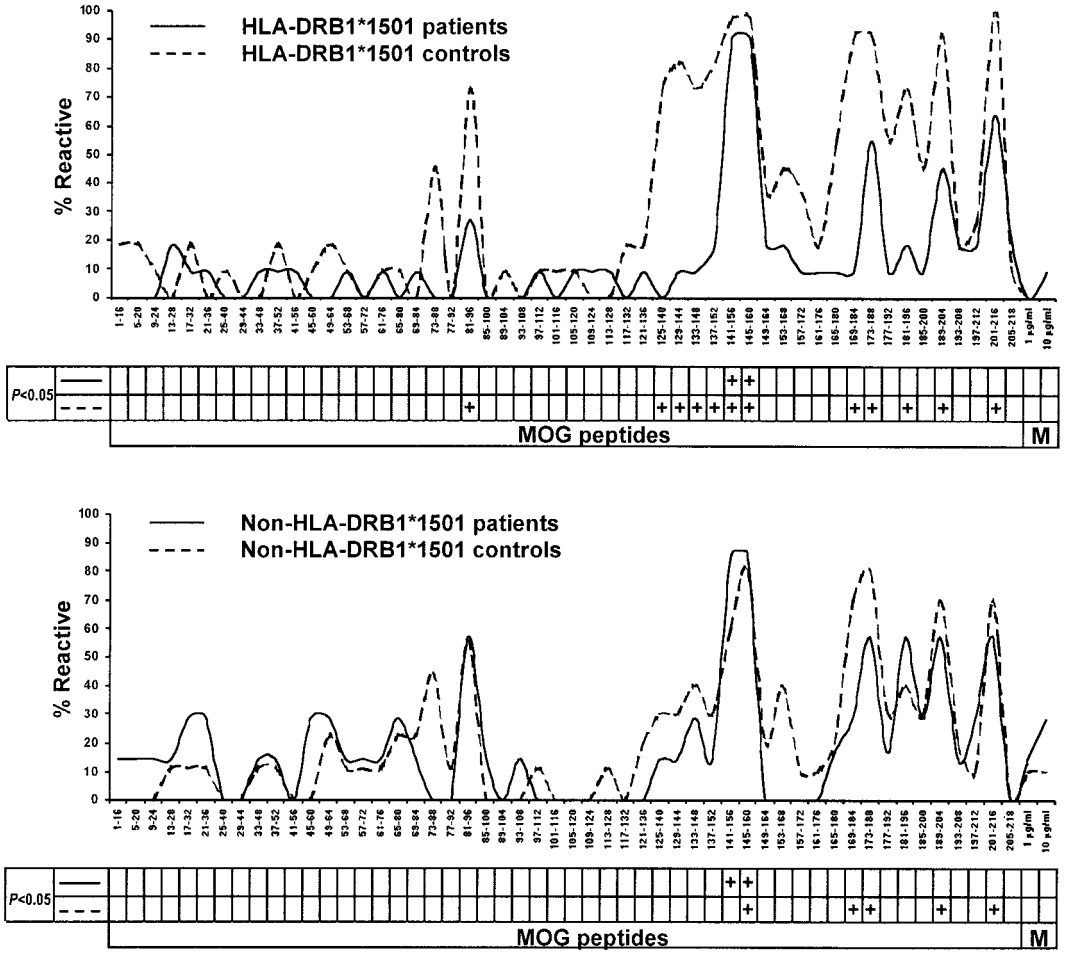

FIGURE 2. Reactivity profiles of MS patients and controls toward overlapping peptides of the complete human MOG sequence. Reactivity profiles of PBMCs secreting IFN-γ in ELISPOT assay toward overlapping MOG peptides and rhMOG 1–125 in 11 DRB1*1501 positive MS patients, 10 DRB1*1501 positive healthy controls, 7 DRB1*1501 negative MS patients, and 10 DRB1*1501 negative healthy controls are indicated. Data represent percentage of reactivity per group toward the respective Ags. Reactivity per patient and per Ag was scored positive if the number of spots per determination was higher than twice the individual background reactivity. Repeated measures ANOVA on signed ranks were used with the Dunnett's test for each of the four groups independently (DRB1*1501 positive MS patients, DRB1*1501 positive controls, DRB1*1501 negative MS patients, DRB1*1501 negative controls). A value of $p < 0.05$ indicates statistical significance after multiple comparisons within each group. In general, the intracellular part of MOG 123–218 was more immunogenic than the extracellular part (MOG 1–122) and controls showed a broadened reactivity profile toward MOG compared with MS patients. The numbering of peptides refers to the MOG sequence. M indicates rhMOG 1–125. The procedures were performed as described in Materials and Methods.

Background values for nonstimulated cultures did not differ significantly between MS patients and controls (NS) (data not shown).

T cell repertoire analysis to the transmembrane and intracellular part of MOG

Compared with the extracellular part of MOG, more reactive T cells were present to the transmembrane and intracellular part of MOG in MS patients and controls (Table I, Figs. 1 and 2). DRB1*1501 positive controls reacted strongly to several peptides predominantly in the region MOG 125–160 (peptides MOG 125–140, $p < 0.05$; MOG 129–144, $p < 0.05$; MOG 133–148, $p < 0.05$; MOG 137–152, $p < 0.05$; MOG 141–156, $p < 0.05$; and MOG 145–160, $p < 0.05$), region MOG 169–188 (peptide MOG 169–184, $p < 0.05$; MOG 173–188, $p < 0.05$), peptide MOG 181–196 ($p < 0.05$), peptide MOG 189–204 ($p < 0.05$), and peptide MOG 201–216 ($p < 0.05$). In contrast, HLA-DRB1*1501 positive patients showed a focused response toward MOG region 141–160 (peptides MOG 141–156, $p < 0.05$; MOG 145–160, $p < 0.05$). HLA-DRB1*1501 negative controls showed responses to MOG peptide 145–160 ($p < 0.05$), MOG region 169–188 (peptides MOG 169–184, $p < 0.05$; MOG 173–188, $p < 0.05$), MOG peptide 189–204 ($p < 0.05$), and MOG peptide 201–216 ($p < 0.05$). Like HLA-DRB1*1501 positive MS patients, HLA-DRB1*1501 negative patients had a focused response to MOG region 141–160 (peptides MOG 141–156, $p < 0.05$; MOG 145–160, $p < 0.05$). MS patients and controls showed quantitatively dominant reactivities to MOG region 141–160 (peptides MOG 141–156 and MOG 145–160) with up to 423 IFN-γ-secreting cells per 2×10^5 PBMC (0.2%) (Fig. 1, individual quantitative data shown for one HLA-DRB1*1501 positive patient). All investigated subjects showed T cell responses to PHA of at least 1000 spots per 2×10^5 PBMC (data not shown).

MOG 146–154 is the dominant T cell determinant

We analyzed the region within MOG 141–160 more in detail with overlapping nine amino acid-long *N*-acetylated C-amidated peptides (Table II, Fig. 3). The *N*-acetylated nine amino acid-long peptides bind to MHC class II molecules, but not to MHC class I molecules due to the *N*-acetyl group. This analysis revealed a dominant T cell response toward peptide MOG 146–154 in HLA-DRB1*1501 positive MS patients and controls (both $p < 0.05$).

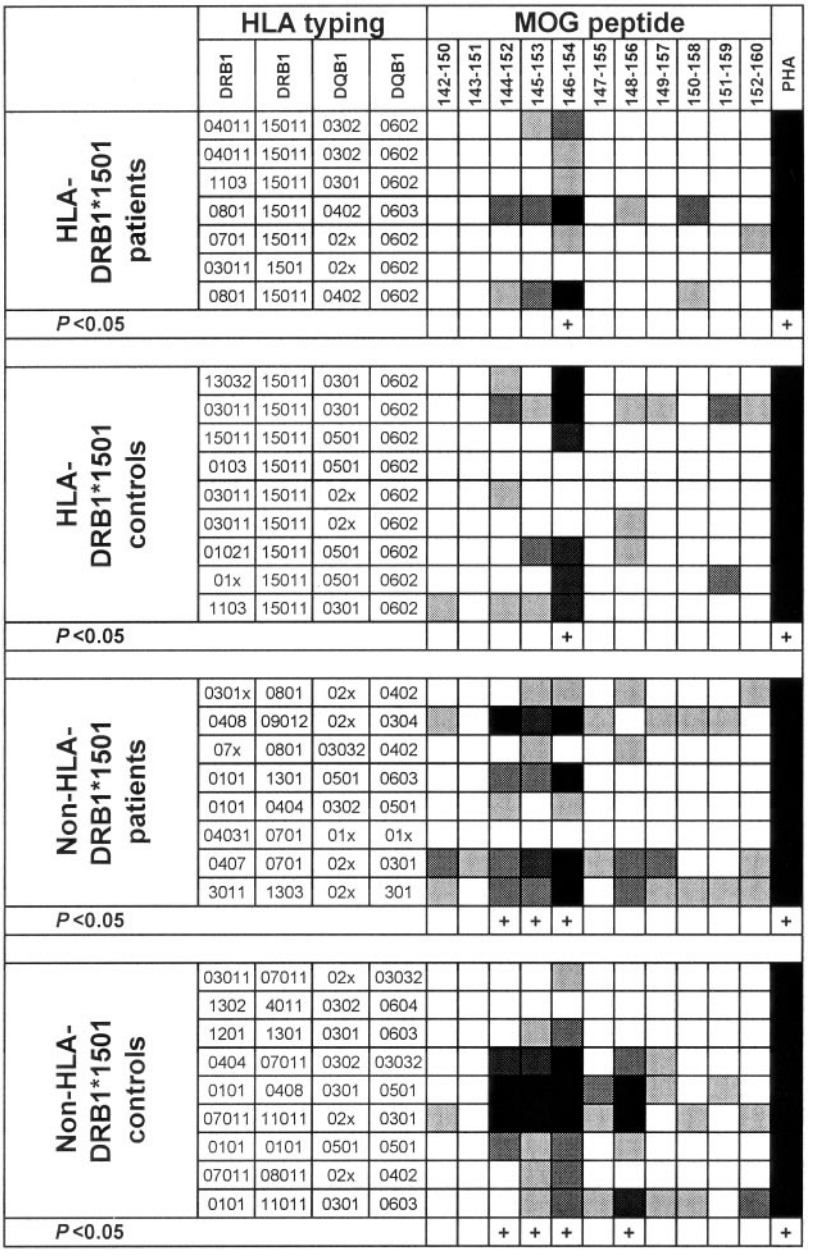

FIGURE 3. Determination of the main T cell determinant within MOG 142–160. Reactivities of PBMCs secreting IFN-γ in ELISPOT assay toward nine amino acid-long peptides of the main immunogenic MOG region MOG 142–160 are shown in seven HLA-DRB1*1501 positive MS patients, nine HLA-DRB1*1501 positive controls, eight HLA-DRB1*1501 negative MS controls, and nine HLA-DRB1*1501 negative controls. Data represent reactivity per patient toward the respective Ags. Reactivity per patient and per peptide was scored positive if the number of spots per determination was higher than twice individual background reactivity (light gray boxes), triple individual background reactivity (gray boxes), four times individual background reactivity (dark gray boxes), or five times individual background reactivity (black boxes). Repeated measures ANOVA on signed ranks were used with the Dunnett's test for each of the four groups independently (DRB1*1501 positive MS patients, DRB1*1501 positive controls, DRB1*1501 negative MS patients, DRB1*1501 negative controls). A value of $p < 0.05$ indicates statistical significance after multiple comparisons within each group. MOG 146–154 was the dominant T cell epitope in DRB1*1501 positive MS patients and controls. Non-HLA-DRB1*1501 MS patients and controls showed a more promiscuous reactivity pattern. MS patients reacted to three peptides and controls to four peptides. The numbering of peptides refers to the MOG sequence. The procedures were performed as described in *Materials and Methods*.

There were broader T cell reactivities in HLA-DRB1*1501 negative patients (peptides MOG 144–152, $p < 0.05$; MOG 145–153, $p < 0.05$; and MOG 146–154, $p < 0.05$) and controls (peptides MOG 144–152, $p < 0.05$; MOG 145–153, $p < 0.05$; MOG 146–154, $p < 0.05$; and MOG 148–156, $p < 0.05$), indicating that the T cell epitopes might be slightly shifted within MOG 141–160 depending on the HLA molecules present. To exclude an influence of disulfide bonds or posttranslational modifications of cysteine (C), we investigated the T cell responses with a modified peptide containing valine (V) instead of cysteine (C) in position 148 (Ac-LVFL*C*LQYR-NH_2→Ac-LVFL*V*LQYR-NH_2). There was no decreased reactivity to this peptide in MS patients or controls (data not shown).

T cell reactivity to influenza A peptide

To exclude a generally decreased immune reactivity in MS patients, we measured T cell reactivities to an influenza A peptide (YRNLVWFIKKNTRYP) (18), with ELISPOT assay for IFN-γ secreting cells. MS patients and controls reacted similarly to this peptide arguing against a generally compromised immune status in MS patients (NS) (data not shown).

Phenotypic analysis of MOG 146–154 reactive cells

To determine the phenotype of IFN-γ-secreting MOG 146–154 reactive T cells, we performed enrichment of these cells and subsequent FACS analysis for expression of CD4 or CD8 and IFN-γ. MOG 146–154-specific T cells expressing IFN-γ were enriched by the IFN-γ enrichment assay in three MS patients (two HLA-DRB1*1501 positive, one HLA-DRB1*1501 negative) and one control (HLA-DRB1*1501 positive). The MOG 146–154-reactive IFN-γ-secreting T cells were of the CD4 phenotype (Fig. 4).

Restriction analysis of MOG 146–154-reactive cells

The restriction pattern of MOG 146–154 reactive T cells was assessed in a HLA-DRB1*1501 positive and a negative MS patient and a HLA-DRB1*1501 positive control. The T cell response was HLA-DR restricted in the investigated HLA-DRB1*0701 DRB1*15011 DQB1*02× DQB1*0602 MS patient and in the

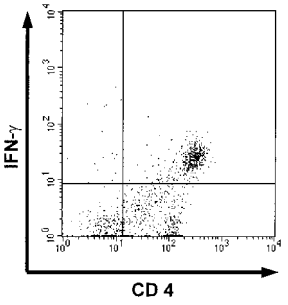

FIGURE 4. MOG 146–154 IFN-γ-secreting T cells are of the CD4 phenotype. Cells secreting IFN-γ after contact with MOG 146–154 on MHC class II molecules of APC were enriched by IFN-γ secretion assay based on an affinity matrix on the cell surface that binds secreted IFN-γ. Subsequently, cells were detected by a secondary PE-labeled Ab against IFN-γ and then separated from cells not having secreted IFN-γ by PE-labeled microbeads in a magnetic field. Isolated cells were stained for IFN-γ and CD4 or CD8 expression and analyzed by FACS. MOG 146–154 reactive IFN-γ-secreting cells of all investigated MS patients ($n = 3$) and a control ($n = 1$) were of the CD4 phenotype. The procedures were performed as described in *Materials and Methods*.

HLA-DRB1*1103 DRB1*15011 DQB1*0301 DQB1*0602 control as well as in a HLA-DRB1*0701 DRB1*0407 DQB1*02× DQB1*0301 MS patient, because only anti-HLA-DR Abs (Tü 36) resulted in decreased T cell responses as compared with addition of anti-HLA-DQ Abs (Genox) (Fig. 5).

*Binding of MOG 141–160-derived peptides to purified HLA-DRB1*1501 and HLA-DRB1*0401 molecules*

We assessed binding affinities of MOG 141–156, MOG 145–160, the complete set of single stepped MOG peptides covering the region MOG 141–160, MBP 85–99, and influenza A peptide to purified HLA-DRB1*1501 and HLA-DRB1*0401 molecules. MOG 141–156 and MOG 145–160, as well as influenza A peptide and MBP 85–99 bound to both alleles. The strength of the binding for these peptides differed in both alleles with HLA-DRB1*1501 binding all four peptides with a higher affinity as indicated by a lower IC_{50} and a higher $1/IC_{50}$ (Fig. 6). Large differences emerged in the binding patterns of the shorter N-acetylated C-amidated nine amino acid-long stepped MOG peptides. HLA-DRB1*1501 molecules bound all these peptides with low to high affinity, depending on the peptide sequence (Fig. 6). In contrast, HLA-DRB1*0401 only bound peptides MOG 144–152, MOG 145–153, and MOG 146–154 with a weak affinity (Fig. 6). Within the set of overlapping single stepped nine amino acid-long MOG peptides, intermediate to high affinity binding values were obtained for MOG 145–153, MOG 146–154, MOG 148–156, MOG 150–158, and MOG 151–159 for HLA-DRB1*1501 molecules. The highest affinity values for HLA-DRB1*0401 molecules, representing weak affinities, were obtained for MOG 144–152, MOG 145–153, and MOG 146–154. Taken together, this data indicates allele-specific differences in the binding of MOG peptides to HLA-DRB1*1501 and HLA-DRB1*0401 molecules with more peptides binding with a higher affinity to HLA-DRB1*1501 molecules. MOG 146–154 had similar to slightly better affinity than MBP 85–99 for HLA-DRB1*1501 or DRB1*0401 molecules. MBP 85–99 had been shown to be the major HLA-DRB1*1501-restricted T cell epitope in MS patients, but is also recognized by T cells in context with several other HLA-DR molecules (19–23).

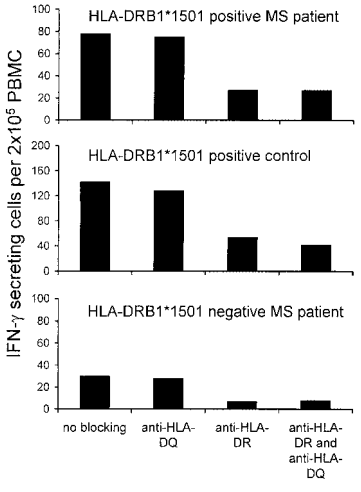

FIGURE 5. Restriction analysis by ELISPOT for IFN-γ-secreting cells toward MOG 146–154. The restriction of the T cell response toward MOG 146–154 is shown for three individuals. The T cell response toward MOG 146–154 was HLA-DR restricted in a HLA-DRB1*0701 DRB1*15011 DQB1*02× DQB1*0602 MS patient (*upper panel*) and in the HLA-DRB1*1103 DRB1*15011 DQB1*0301 DQB1*0602 control (*middle panel*), as well as in a HLA-DRB1*0701 DRB1*0407 DQB1*02× DQB1*0301 MS patient (*lower panel*). Blocking studies were performed with the mAbs Genox (anti-HLA-DQ) and/or Tü 36 (anti-HLA-DR). Collection of cells, blocking studies, and evaluation of data were performed as described in *Materials and Methods*.

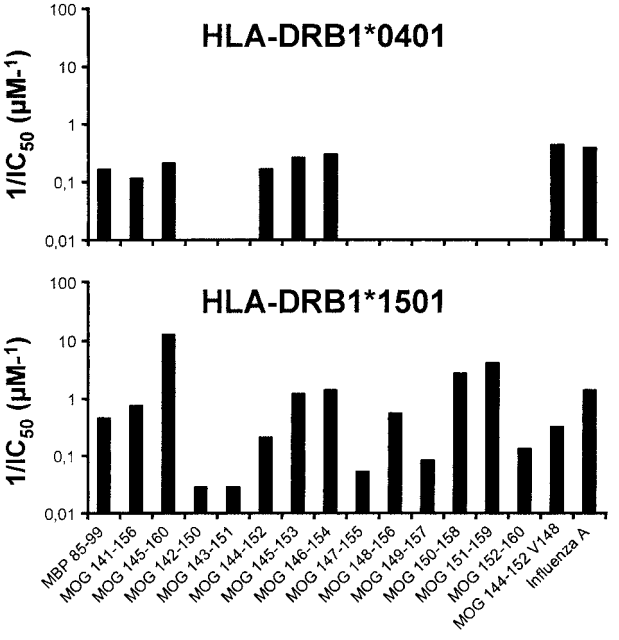

FIGURE 6. In vitro peptide binding profiles of MOG 141–160-derived peptides to HLA-DRB1*1501 and HLA-DRB*0401 molecules. Binding affinities of MOG 141–156 and MOG 145–160, MBP 85–99, influenza A peptide, and nine amino acid-long MOG peptides of MOG sequence MOG 142–160 were measured to purified HLA-DRB1*1501 and HLA-DRB1*0401 molecules with a competitive binding assay and detection by the fluorochrome europium. The number of binding MOG peptides and the strength of binding differed for HLA-DRB1*1501 and HLA-DRB1*0401 molecules. MOG 141–156, MOG 145–160, MBP 85–99, and influenza A peptide bound to both molecules with varying affinities. For all nine amino acid-long peptides covering sequence MOG 142–160, binding was detected to HLA-DRB1*1501 molecules (*lower panel*). In contrast, HLA-DRB1*0401 molecules bound MOG 144–152, MOG 145–153, and MOG 146–154 (*upper panel*). MOG 146–154 bound equal or slightly better to HLA-DRB1*1501 or HLA-DRB1*0401 molecules compared with MBP 85–99. The procedures were performed as described in *Materials and Methods*.

Discussion

This study demonstrates for the first time that 1) there is a dominant MOG epitope recognized by $CD4^+$ T cells within the intracellular part of MOG comprising aa 146–154 in MS patients and controls; 2) MS patients show a more focused T cell reactivity pattern toward MOG compared with controls; 3) the dominant MOG T cell epitope is promiscuously recognized in context of several MHC class II molecules; and 4) the intracellular part of MOG is much more immunogenic compared with the extracellular part.

In animal models with marmosets, rats, and mice, the extracellular part of MOG leads to a very MS-like disease (7, 24–26) (Fig. 7). In addition, Amor et al. (25) investigated the encephalitogenic potential of peptides derived from the transmembrane and intracellular part of MOG in Biozzi AB/H and SJL mice. Our study is the first systematic study investigating T cell responses in MS patients and controls to peptides of the complete MOG sequence. Interestingly, besides the T cell responses to MOG 81–96, only minor T cell responses to the extracellular part of MOG were detectable. In contrast, several stretches of the transmembrane/intracellular domain of MOG proved to be highly immunogenic. We defined an immunodominant T cell epitope encompassing MOG 146–154 with *N*-acetylated and C-amidated nine amino acid-long peptides that can bind to MHC class II molecules. There was no major HLA-guided influence on the selection of T cells arguing for promiscuous presentation of this MOG peptide on MHC class II molecules. In the tested MS patients and controls, the MOG 146–154-specific T cell response was HLA-DR restricted. We assessed in vitro binding affinities of MOG peptides spanning MOG sequence 141–160 to two purified HLA-DR molecules. Both HLA-DRB1*1501 as well as HLA-DRB1*0401 molecules bound MOG 146–154 to a varying degree allowing presentation of this peptide to T cells. Interestingly, HLA-DRB1*1501 molecules bound more of the nine amino acid-long MOG peptides within the sequence MOG 142–160 compared with HLA-DRB1*0401 molecules. Both alleles bound MOG 146–154 slightly better than MBP 85–99 that is the major HLA-DRB1*1501-restricted MBP stretch in MS patients (19–23).

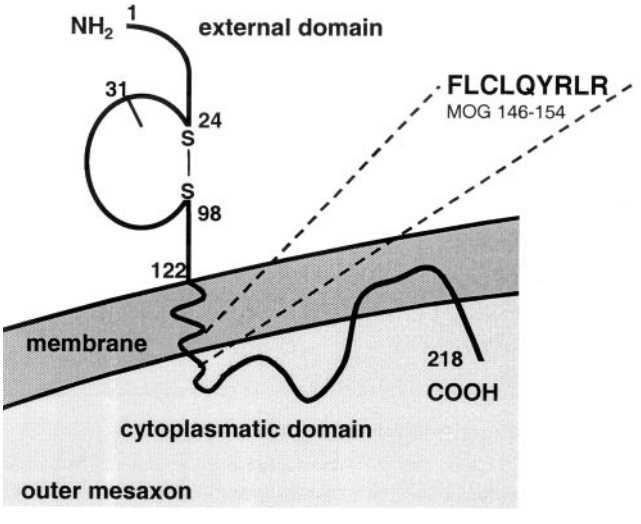

FIGURE 7. Schematic view of the MOG molecule. MOG is exposed on the extracellular surface of the myelin sheath. The extracellular part has an Ig-like structure and composes aa 1–122. It is glycosylated at position 31. The transmembrane part is composed of aa 122–150 and the cytoplasmatic domain of aa 150–218. Major T cell reactivity was detected against the transmembrane and cytoplasmatic domain in MS patients and controls. MOG 146–154 was dominantly recognized in MS patients and controls. MOG model according to Della Gaspera et al. (11).

Our results of promiscuous recognition of MOG 146–154 by T cells in context of several MHC class II molecules are partly in line with T cell reactivity to MBP in MS patients and controls. The immunodominant MBP 84–102 and MBP 87–106 peptides and their core sequence MBP 89–99 are recognized in the context of several MHC molecules in man as well as in mice and rats (19–23, 27). This MBP stretch induces experimental autoimmune encephalomyelitis (EAE) in HLA-DR2 transgenic mice (28). In future studies, our laboratories will assess the immunogenic and encephalitogenic potential of intracellular MOG determinants in animal models.

Epitope mapping studies in humans with regard to myelin-Ags have shown conflicting results as far as reactivity patterns of T cell responses in diseased individuals compared with controls are concerned. Some studies have shown that there is an increased frequency of autoreactive T cells from blood in diseased individuals and that T cell reactivity is increased in MS patients, while others recorded negative findings in regard with disease-associated differences in blood (12, 23, 29). Especially IFN-γ-secreting cells have been shown to be elevated in diseased individuals in blood and cerebrospinal fluid (30). Our study is partly contradictory to these observations because controls showed even more T cell reactivities in blood ex vivo compared with MS patients. Importantly, we did not find a generally compromised immune status in regard to PBMC reactivity in MS patients that would have explained this finding, because T cells from blood reacted similar to an influenza A peptide in MS patients and controls (18).

We did not observe the same reactivity profile to the extracellular part of MOG as Wallström et al. (12) had demonstrated with MOG 63–87 being immunodominant. Reasons might lie in differences in the genetic background of the patients and the preceding Ag exposure with effects on the T cell repertoire in the Swedish compared with the German MS population (31). Additionally, compared with the explicit strength of the T cell responses to MOG 81–96 in some MS patients and controls and intracellular MOG in our study, in this preceding study the T cell responses as assessed by IFN-γ ELISPOT were very weak with 3–4 spots in mean per 1×10^5 PBMC to the immunodominant MOG 63–87. In this study, beside MOG 63–87 also MOG 76–100 was slightly more recognized by MS patients compared with controls (1–2 spots per 1×10^5 PBMC) (12). The peptide MOG 79–96 has been shown to induce severe disease in DBA/1 ($H\text{-}2^q$) mice (32) and mild disease in LEW.1AV1 ($RT1^{av1}$) and LEW.1N ($RT1^n$) rats (26). Interestingly, we did not find T cell responses in humans to peptides MOG 89–104, MOG 93–108, MOG 97–108, and MOG 97–112. These peptides contain the major encephalitogenic MOG stretches MOG 92–106 in SJL/J ($H\text{-}2^s$) mice (25), MOG 97–108 in HLA-DRB1*0401 transgenic mice (33), and MOG 91–108 in different inbred rat strains, bearing the $RT1^a$, $RT1^{av1}$, and $RT1^n$ haplotypes, respectively (26). Nor did we detect strong T cell responses to peptides MOG 33–48 and MOG 37–52 that contain the encephalitogenic MOG determinant MOG 35–55 of C57BL/6 ($H\text{-}2^b$) and NOD/LT ($H\text{-}2^{g7}$) mice (6). In Biozzi AB/H ($H\text{-}2^{dq1}$) mice, MOG 134–148 induced mild signs of EAE in one of five mice (25). The same authors did not detect encephalitogenic sequences in Biozzi AB/H ($H\text{-}2^{dq1}$) and SJL ($H\text{-}2^s$) mice to overlapping peptides covering MOG 141–218 (25). The data underscores the need to evaluate encephalitogenic MOG responses in humanized animal models additionally to inbred mouse and rat strains (34).

In MOG-induced EAE in LEW.1N rats, we have recently shown that MHC class II-regulated CNS autoaggression and T cell responses in peripheral lymphoid tissues are dissociated (26). The major encephalitogenic MOG 91–108 peptide in LEW.1N rats did not induce a detectable proliferative response and T1 or T2 T cell

response in lymph nodes or spleen ex vivo after active immunization with MOG 91–108 or MOG 1–125. Instead, in the target tissue, the CNS, strong cellular, and cytokine responses were present after immunization with this peptide (26). In contrast, there were several determinants within MOG 1–125 that did raise strong T cell responses in peripheral lymphoid tissue, but did not induce disease (26). These data point to the need to investigate disease-associated cellular responses within the target organ (35). Unfortunately, investigations of the intra-CNS immune response in humans are difficult to perform because the availability of cerebrospinal fluid is limited and there are a number of ethical problems.

There was a focusing of the T cell reactivity patterns toward MOG 141–160 in MS patients. The decreased number of T cell determinants in MS patients could reflect alteration of the T cell repertoire by preceding Ag exposure after damage of the blood brain barrier leading to a state of peripheral T cell tolerance to certain MOG determinants (36). Moreover, migration of encephalitogenic T cells in MS patients to the target organ and reduction of the size of the T cell pool in blood reactive with MOG cannot be excluded at present (3, 37). Such a scenario would argue for a pathogenic role of the T cell reactivities that are absent or reduced in the blood of MS patients compared with controls in the disease process and again forces investigations at the target organ site. Alternatively, other functional differences might exist between the MOG reactive T cells secreting IFN-γ of MS patients and controls. Moreover, regulatory mechanisms could be up-regulated in MS patients after establishment of disease reducing the reactivity profile of T cells to MOG determinants in blood and lymphoid organs like up-regulation of TGF-β (38) or increased NK cell reactivity (39).

Our data indicate that during thymic selection, MOG 146–154 cross-reactive T cells must be positively selected on several HLA class II molecules and not deleted. In EAE, it has been recently shown that intrathymic expression of myelin components can lead to tolerance (40). Compared with other proteins of the myelin sheath, the expression of MOG is low because it comprises only 0.01% of the protein content of the myelin sheath (6). To date, no expression of human MOG on non-CNS tissue including the thymus has been reported, but it is not excluded that MOG is expressed at a very low level in the thymus. Potentially, this absent or low level expression would allow escape of MOG-specific T cells from negative selection in the thymus (40). The extracellular part of MOG shows homology to butyrophilins and B7 family members that are expressed in the thymus (41). In rats, butyrophilins have been shown to be cross-reactive with the extracellular part of MOG (42). In contrast, structural similarities between self-proteins and the transmembrane or intracellular domain of MOG have not been described to the same degree as to the extracellular part of MOG. As a consequence, central tolerance mechanisms might result in more effective negative selection of T cells reactive to the extracellular part of MOG compared with the intracellular part. This would explain the high number of IFN-γ-secreting cells against the intracellular part of MOG present in the periphery.

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