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Molecular and Genetic Requirements for Preferential Recruitment of TCRBV8S2⁺ T Cells in Lewis Rat Experimental Autoimmune Encephalomyelitis

Robert Weissert,* 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), LEW.1AV (RT1av), and LEW.1W (RT1w)) 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,RA,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,RA,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⁺ 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.

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E xperimental autoimmune encephalomyelitis (EAE) 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 TCRBV 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, MBP,GP,89-101, 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 MBP,63-88 peptides, and, secondly,
to quantify the preferential recruitment of TCRBV8S2+ T cells. Inbred MHC congenic LEW rats (LEW(RT1u), LEW.1AV1(RT1av1), and LEW.1W(RT1w)) 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 (MBPGP63-88) and, in addition, in LEW rats immunized with the corresponding autologous rat MBP peptide (MBPRAT63-88). 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 MBPGR63-88 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 (RT1av1), and LEW.1W (RT1w) 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

MBPGR63-88 (AARTTHYGSLPQKSRQTDENPVVFH), MBPRAT63-88 (HTRTTHYGSLPQKSRQTDENPVVFH), and MBPGR68-101 (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 MBPGR72-85 (LQPQKSRQDENPV) 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 TCRβ (clone R73, mouse IgG1k), phycoerythrin (PE)-labeled anti-rat TCRBV8S2 (clone R78, mouse IgG1k), PE-labeled anti-rat TCRBV10 (clone G101, mouse IgG2ak), FITC-labeled anti-rat CD25 (IL-2Rα-chain, clone WT.1, mouse IgG2ak), FITC-labeled mouse IgG1k (clone 107.3), and PE-labeled mouse IgG2ak (clone G155-178). The anti-TCRBV8S2 mAb (clone R78, mouse IgG1k) 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 MBPGR63-88 or MBPRA63-88 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 NH4Cl, 1 mM KHCO3, and 0.1 mM Na2EDTA adjusted to pH 7.4.

MNC were cultured at a concentration of 2 × 106 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% CO2. For each rat/Ag combination, MNC were cultured for 72 h, washed twice in DMEM, adjusted to a concentration of 20 × 106 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 × 106 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 interface, 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 × 105 MNC/well were cultivated in CM with or without the relevant Ags for 60 h and, subsequently, pulsed with 0.5 μCi of [3H]Tdr (Amersham, Buckinghamshire, U.K.) per well for an additional 12 h. DNA was collected on glass fiber filters (Skatron, Sterling, VA) and [3H]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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Haplotypes</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
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<td>LEW</td>
<td>I</td>
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<td>I</td>
<td>L</td>
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<tr>
<td>LEW.1AV1 (DA)a</td>
<td>av1</td>
<td>a</td>
<td>a</td>
<td>av1</td>
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<tr>
<td>LEW.1W (MP)</td>
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a Donor strains are indicated within parentheses.
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 × 10^3 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% CO₂. 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⁺ molecules

RT1.B⁺ 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 MBP₆₃₋₈₈ (200 nM) was used as the reference peptide. Briefly, RT1.B⁺ molecules (200 nM) were incubated with the reference peptide (500 nM) in the presence of various concentrations of each of the unlabeled relevant peptides, MBP₆₃₋₈₈, MBP_{RAT}₆₃₋₈₈, and MBP_{GP/RAT}₈₉₋₁₀₁. The binding buffer consisted of 2 mM EDTA, 25 mM Na₂CO₃, 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 (IC₅₀). IC₅₀ 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.

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. 1A). Both rat strains generated a self-limiting monophasic disease profile, reaching a mean maximum disease score of grade 3. The semi-resistant 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}₆₈₋₈₈ (Fig. 1B). 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.
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<sub>GP</sub>63-88-primed LEW rats, the number of TCRBV8S2<sup>+</sup> T cells co-expressing CD25 was significantly increased (p < 0.0003), whereas the frequency of TCRBV10<sup>+</sup>/CD25<sup>+</sup> T cells was unaffected (Fig. 2). Such a selective, increased expression of CD25 in the TCRBV8S2<sup>+</sup> T cell subset was not observed in LEW rats immunized with MBP<sub>RA</sub>63-88. Likewise, MBP<sub>GP</sub>63-88-primed LEW.1AV1 and LEW.1W rats, CFA-injected, and naive controls exhibited unchanged frequencies of both the TCRBV8S2<sup>+</sup>/CD25<sup>+</sup> and the TCRBV10<sup>+</sup>/CD25<sup>+</sup> T cell subset. Due to the delayed onset of clinical signs in the LEW.1W strain (Fig. 1A), we assessed CD25 expression on TCRBV8S2<sup>+</sup> T cells also on day 16 p.i. No preferential activation was observed (data not shown).

Expansions of peripheral TCRBV8S2<sup>+</sup> T lymphocytes

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

Frequencies of CNS-infiltrating TCRBV8S2<sup>+</sup> T lymphocytes

In view of the over-representation of activated TCRBV8S2<sup>+</sup> T cells in blood from MBP<sub>GP</sub>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<sup>+</sup> T cells was thus determined at the site at which the autoantigenic MBP target molecule is exposed. LEW, LEW.1AV1, and LEW.1W rats

![Image](http://www.jimmunol.org/)

**FIGURE 2.** Frequencies of activated peripheral TCRBV8S2<sup>+</sup> T cells from MBP63-88-primed RT1 congenic LEW rat strains. A illustrates typical two-color flow cytometric analyses of TCRBV8S2<sup>+</sup>/CD25<sup>+</sup> and TCRBV10<sup>+</sup>/CD25<sup>+</sup> T cells from draining LN of a randomly chosen individual from the groups of RT1 congenic LEW rats immunized with MBP<sub>GP</sub>63-88 and from LEW rats immunized with MBP<sub>RA</sub>63-88, mentioned in B. B depicts the frequencies of TCRBV8S2<sup>+</sup>/CD25<sup>+</sup> and TCRBV10<sup>+</sup>/CD25<sup>+</sup> T cells from blood and draining LN of MBP<sub>GP</sub>63-88-primed LEW (n = 7), LEW.1AV1 (n = 5), and LEW.1W (n = 4) rats as well as MBP<sub>RA</sub>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).
were immunized with MBP<sub>gp</sub>63-88 or MBP<sub>rat</sub>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<sub>gp</sub>63-88 expressed TCRBV8S2 (p<0.0003) (Fig. 4). This relative frequency of CNS-associated TCRBV8S2<sup>+</sup> 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<sup>+</sup> T lymphocytes was recorded in LEW rats immunized with the autologous MBP<sub>rat</sub>63-88 peptide, or LEW.1AV1 and LEW.1W rats immunized with MBP<sub>gp</sub>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).

**FIGURE 3.** Expansions of peripheral TCRBV8S2<sup>+</sup> T lymphocytes in MBP<sub>gp</sub>63-88-primed LEW rats. A illustrates typical two-color flow cytometric analyses of TCRBV8S2/TCR(A/B)<sup>+</sup> and TCRBV10<sup>+</sup>/TCR(A/B)<sup>+</sup> T cells from the blood of a randomly chosen individual from the groups of LEW rats immunized with either MBP<sub>gp</sub>63-88, MBP<sub>rat</sub>63-88, or CFA alone and from a naïve animal, mentioned in B. B indicates the frequencies of TCRBV8S2/TCR(A/B)<sup>+</sup> and TCRBV10<sup>+</sup>/TCR(A/B)<sup>+</sup> T cells from blood and draining LN of LEW (n=7), LEW.1AV1 (n=5), and LEW.1W (n=4) rats immunized with MBP<sub>gp</sub>63-88 and LEW rats (n=5) immunized with MBP<sub>rat</sub>63-88, as determined by two-color FACS analyses. CFA-injected LEW (n=4) and naïve 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).

**T lymphocyte cross-reactivity with MBP<sub>gp</sub>63-88 and MBP<sub>rat</sub>63-88 in differentially primed LEW rats**

Since LEW rats had shown marked differences after immunization with either MBP<sub>gp</sub>63-88 or MBP<sub>rat</sub>63-88 in the preferential activation and recruitment of TCRBV8S2<sup>+</sup> 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<sub>gp</sub>63-88 or MBP<sub>rat</sub>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<sup>+</sup> and TCRBV10<sup>+</sup> T cells co-expressed CD25 (data not shown). After immunization with MBP<sub>gp</sub>63-88 and in vitro restimulation with the same peptide, the number of TCRBV8S2<sup>+</sup> T cells co-expressing CD25 was selectively increased as opposed to that of the TCRBV10<sup>+</sup>/CD25<sup>+</sup> T cell subset (p<0.01). Interestingly, this was also true, but to a lower extent, after culture with MBP<sub>rat</sub>63-88 (p<0.05) (Fig. 5A). Thus, TCRBV8S2<sup>+</sup> T cells preferentially expanded due to the heterologous MBP<sub>gp</sub>63-88 peptide, which could recognize and maintain an increased CD25 expression in response to the syngeneic MBP<sub>rat</sub>63-88 peptide. No significant changes in expression of CD25 on TCRBV8S2<sup>+</sup> cells were recorded after secondary stimulation in vitro with MBP<sub>rat</sub>63-88 and MBP<sub>gp</sub>63-88 of cells from MBP<sub>rat</sub>63-88-primed LEW rats (Fig. 5B).

T cells from MBP<sub>gp</sub>63-88-primed LEW rats displayed a proliferative response both to MBP<sub>gp</sub>63-88 and MBP<sub>rat</sub>63-88, with the former eliciting a slightly higher response (Fig. 6A). T cells from MBP<sub>rat</sub>63-88-primed LEW rats proliferated roughly at similar
levels after culture with MBP GP 63-88 and MBP RAT 63-88 (Fig. 6B). Even more conspicuous and discriminating MBP peptide responses were recorded by using production of the proinflammatory cytokine IFN-\(\gamma\) as the outread. Lymphoid cells from MBP GP 63-88-immunized LEW rats showed high numbers of IFN-\(\gamma\)-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. 6C). MBP RAT 63-88-immunized LEW rats had high numbers of IFN-\(\gamma\)-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. 6D).

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-TCRV8S2 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

**Materials and Methods.** Each bar represents the mean ± SEM. ** and * indicate statistical significance (\(p < 0.01\) and \(p < 0.05\)).
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 MBPRAT 63-88 (\(p\) \(\leq 0.05\)) and LEW.1AV1 rats pre-immunized with MBP\(_{GP}\) 63-88 (\(p\) \(\leq 0.05\)) (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.Bl molecules (Fig. 8). RT1.Bl 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 \(\mu\)M). Practically equally strong inhibition of binding was observed for both MBP63-88 peptides (IC\(_{50}\) MBP\(_{GP}\) 63-88, 2.5 \(\mu\)M; MBP\(_{RAT}\) 63-88, 0.7 \(\mu\)M). In comparison, the MBP\(_{RAT}\) 89-101 peptide, which has been reported to associate with the RT1.D\(^{l}\)

FIGURE 6. Ag-specific proliferation and enumeration of IFN-\(\gamma\)-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-\(\gamma\)-secreting cells per 4 \(\times\) 10\(^{3}\) 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-\(\gamma\)-secreting cells \(\pm\) SEM.

FIGURE 7. In vivo depletion of TCRBV8S2\(^{+}\) T cells. All RT1 congenic LEW rat strains were initially immunized with 200 \(\mu\)g of the relevant MBP63-88 peptides, followed by injection with 150 \(\mu\)g 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 (\(p < 0.05\)).
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 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.B1 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, T79 and S79, both appear to qualify as anchor residues for pocket 6 of the allele-specific RT1.B1 groove. However, another recently published study, in which a panel of alanine-substituted MBP67-85 analogues was tested for each member’s relative binding strength to the RT1.B1 motif, indicated that T79 and S79 would rather act solely as TCR contact residues (38). Nevertheless, despite the fact that the single T79/S79 substitution has to be considered conservative, and that competitive binding studies of MBP63-88 and MBP63-88 excluded differences in their affinities for the RT1.B1 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.B1 complex. On the assumption that T79 and S79 interact with pocket 6 of the RT1.B1 groove, their differential contribution to the three-dimensional structure of the peptide/RT1.B1 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, T79 and/or S79 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 MBP63-88 and MBP63-88, respectively, in the TCR/peptide/RT1.B trimolecular complex awaits its resolution, possibly by virtue of employing x-ray crystallography.
reertoire (42). By the use of inbred MHC congenic LEW rats, kept under identical physical conditions, we can preclude germ-line-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-GP63-88-primed LEW rat.

The capacity for eliciting preferential peripheral activation of MBP-GP63-88-specific TCRBV8S2 " T cells may depend on central tolerance induction. Since only LEW rats with intact thymus exhibit preferential TCRBV8S2 usage (39), it can be presumed that the autoreactive MBP-GP63-88-specific TCR repertoire is shaped to a high degree by self-peptides presented by RT1.B1 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 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.B1 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-GP63-88/self-RT1.B1 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-GP63-88-specific T cells expressing TCRBV8S2 may prevail.

Differences in discordance 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-GP63-88 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 RT1.1-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-GP63-88/RT1.1 class II complex. This series of events may result in autoreactive cross-reactivity with the MBP-GP63-88/RT1.1 class II complex, thus becoming instrumental for induction of EAE. Indeed, we observed that MBP-GP63-88-primed TCRBV8S2 " T cells responded to MBP-GP63-88 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 TCRBV.