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Recombinant TCR ligand reverses clinical signs and CNS damage of EAE induced by recombinant human MOG¹

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Abstract

Increasing evidence suggests that in addition to T cell dependent effector mechanisms, autoantibodies are also involved in the pathogenesis of MS, including demyelinating antibodies specific for myelin oligodendrocyte glycoprotein (MOG). Our previous studies have demonstrated that recombinant T cell receptor ligands (RTLs) are very effective for treating T cell mediated experimental autoimmune encephalomyelitis (EAE). In order to expand the scope of RTL therapy in MS patients, it was of interest to study RTL treatment of EAE involving a demyelinating antibody component. Therefore, we evaluated the therapeutic effects of RTL551, specific for T cells reactive to mouse (m) MOG-35-55 peptide, on EAE induced with recombinant human (rh)MOG in C57BL/6 mice. We report that RTL551 therapy can reverse disease progression and reduce demyelination and axonal damage induced by rhMOG without suppressing the anti-MOG antibody response. This result

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suggests that T cell mediated inflammation and associated blood-brain barrier dysfunction are the central contributors to EAE pathogenesis, and that successful regulation of these key players restricts potential damage by demyelinating antibodies. The results of our study lend support for the use of RTL therapy for treatment of MS subjects whose disease includes inflammatory T cells as well as those with an additional antibody component.

Keywords

EAE; MS; recombinant human MOG; CNS damage

INTRODUCTION

Recombinant TCR ligands (RTLs) containing the membrane distal $\alpha 1 + \beta 1$ domains of class II MHC molecules linked covalently to specific peptides can be used to regulate T cell responses. They act as partial agonists signaling directly through the TCR to inhibit experimental autoimmune encephalomyelitis (EAE) in active and passive myelin basic protein (MBP)-induced monophasic disease in Lewis rats (Burrows et al., 1998; Wang et al., 2003), myelin oligodendrocyte glycoprotein (MOG) peptide-induced chronic EAE in wild type and DR2 transgenic mice (Vandenbark et al., 2003; Sinha et al., 2007) and proteolipid protein (PLP)-induced relapsing remitting EAE in SJL/J mice (Huan et al., 2004). RTL constructs derived from HLA-DR2 (Haines et al., 1996) inhibited activation but promoted IL-10 secretion in human DR2-restricted T cell clones specific for MBP-85–99 or cABL (BCR-ABL b3a2) peptides (Burrows et al., 2001; Chang et al., 2001), and one such DR2 construct containing the MOG-35–55 peptide, RTL1000, is currently under evaluation in a Phase 1 safety trial for use in multiple sclerosis (MS).

Increasing evidence suggests that in addition to T cell dependent effector mechanisms, autoantibodies are also involved in the pathogenesis of MS (Hauser, 2008). The deposition of immunoglobulins and complement components in the majority of actively demyelinating lesions (Storch and Lassmann, 1997; Bruck et al., 2002; Merkler et al., 2006) clearly implicate humoral effector mechanisms in lesion formation, a concept supported by the beneficial effect of plasma exchange in some patients (Kieseier and Hartung, 2003). However, the specificity of clinically-relevant antibodies in MS remains controversial, although MOG may provide an important target for demyelinating autoantibodies in ADEM and some patients with relapsing remitting MS (O'Connor et al., 2007).

MOG was initially identified as a target for demyelinating antibodies, but was subsequently also shown to induce encephalitogenic T cell responses in susceptible species. In MOG-induced models of EAE, a combination of MOG-specific T cell and antibody responses act in synergy to reproduce the complex immunopathology of the MS lesion (Marta et al., 2005). As elegantly demonstrated in C57BL/6 mice immunized with recombinant human MOG (rhMOG) and in SJL/J MOG-92–106 peptide-specific TCR Tg mice with spontaneous EAE, the encephalitogenic T cell response is essential for initiating inflammation and damage to the blood brain barrier (Lyons et al., 2002; Oliver et al., 2003; Pollinger et al., 2009). Only then can MOG-specific antibodies gain access to the CNS to initiate a combination of complement and ADCC-dependent mechanisms that exacerbate demyelination and promote CNS inflammation, resulting in severe clinical disease.

Our studies have demonstrated that RTLs are very effective for treating T cell mediated EAE. In order to expand the scope of RTL therapy in MS patients, it was of interest to study RTL treatment of EAE involving a demyelinating antibody component. Therefore, we evaluated the therapeutic effects of RTL551, a partial agonist specific for T cells reactive to mMOG-35–55

peptide, on EAE induced with rhMOG in C57BL/6 mice. We report that RTL551 therapy can reverse disease progression and reduce demyelination and axonal damage induced by rhMOG without suppressing the anti-MOG antibody response. This result suggests that T cell mediated inflammation and associated blood-brain barrier dysfunction are the central contributors to EAE pathogenesis, and that successful regulation of these key players restricts potential damage by demyelinating antibodies.

MATERIALS AND METHODS

Animals

C57BL/6 male mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 7–8 wk of age. The mice were housed in the Animal Resource Facility at the Portland Veterans Affairs Medical Center (Portland, OR) in accordance with institutional guidelines. The study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the Institutional Animal Care and Use Committee.

Antigens

Human recombinant MOG (rhMOG) was a kind gift from Dr. Claude Bernard (Monash University, Australia). Synthetic human (h)MOG-35-55 peptide and mouse (m)MOG-35-55 peptide were synthesized by NeoMPS, Inc. (San Diego, CA).

Induction of active EAE and treatment with RTL551

Mice immunized with rhMOG or mMOG-35-55 peptide received 100µg of rhMOG or 100µg of mMOG-35-55 peptide in an equal volume of CFA containing 2mg/ml heat killed *M. tuberculosis*. All the mice were also injected with 75ng and 200ng pertussis toxin (Ptx) intraperitoneally on days 0 and 2 relative to immunization. The mice were assessed for signs of EAE according to the following scale: 0, normal; 1, limp tail or mild hindlimb weakness; 2, moderate hindlimb weakness or mild ataxia; 3, moderately severe hindlimb weakness; 4, severe hindlimb weakness or mild forelimb weakness or moderate ataxia; 5, paraplegia with no more than moderate forelimb weakness; and 6, paraplegia with severe forelimb weakness or severe ataxia or moribund condition. At the onset of clinical signs of EAE (days 10–13 when the clinical scores were ≥ 2), mice were divided into two groups and treated with 100µl of 20mM Tris-HCL as controls or with 100µl of 1 mg/ml RTL551 i.v. along with antihistamine for 8 days. No effect of antihistamine has been observed on EAE induction and progression in SJL/J (Huan et al., 2004) or C57BL/6 mice (HO, unpublished). We have shown before that administration of molar equivalent dose of free peptide, PLP139-151 to SJL (5) and MOG35-55 to C57BL/6 (unpublished), with antihistamine had no significant clinical benefit to the mice with EAE as compared to untreated mice. Mice were monitored for changes in disease score and were boosted with the treatments as indicated until they were euthanized for ex vivo analyses. Method for design, cloning and expression of RTL551 has been published elsewhere (Sinha et al., 2007)

Proliferation assays

Draining lymph nodes (LN) were harvested from vehicle- and RTL-treated mice on day 26. A single cell suspension was prepared by homogenizing the tissue through a fine mesh screen. Cells were cultured in a 96-well flat-bottom tissue culture plate at 4×10^5 cells/well in stimulation medium either alone (control) or with indicated test antigens (hMOG-35-55 peptide or rhMOG) at varying concentrations. Cells were incubated for 3 days at 37°C in 7% CO₂. Cells were then pulsed with 0.5µCi of [³H] thymidine (PerkinElmer, Boston, MA) for the final 18 h of incubation and harvested onto glass fiber filters, and tritiated thymidine uptake was

measured by a liquid scintillation counter. Means and SDs were calculated from triplicate wells. The Stimulation Index (SI) was calculated using the following formula: CPM of antigen wells/ CPM of medium alone wells.

Cytokine determination by Luminex Bio-Plex assay kit

Single cell suspensions of spleens (harvested on Days 17 and 26) and LNs (harvested on Day 26) from vehicle and RTL treatment groups were prepared by homogenizing the tissue through a fine mesh screen. Four million cells from vehicle and RTL-treated groups were cultured in the presence of rhMOG in a 24 well tissue culture plate for 48hrs. Culture supernatants were assessed for cytokine levels using a Luminex Bio-Plex cytokine assay kit (Bio-Rad Laboratories, Inc., Richmond, CA) following the manufacturer's instructions. The following cytokines were determined in a single assay in three separate experiments: IL-1 β , IFN- γ , TNF- α , IL-2, IL-4, IL-10, IL-17, IL-5, IL-6, IL-12 IL-13 and MCP-1.

Antibody ELISA

Sera were collected from each group of mice on day 26 post immunization and rhMOG specific Ig (kappa chain), IgG1 and IgG2b Ab levels were measured by ELISA. Additionally, serum samples from mMOG-35-55 immunized mice were included as controls. All the serum samples were subjected to ELISA (on plates coated with rhMOG) before and after adsorption with respective peptides (mMOG-35-55 for mMOG-35-55-immunized mice and hMOG-35-55 for rhMOG-immunized mice). Ninety-six-well plates were coated with 10 μ g/ml rhMOG in PBS and incubated overnight at 4°C. Plates were blocked with blocking buffer (1x PBS, 2% BSA, 0.05% Tween 20) for 2h at 4°C. Plates were washed and 100 μ l of diluted (1:1000) serum sample in blocking buffer was added to triplicate wells. Samples were incubated at 4°C for 1hr. One hundred microliters of 1/5000 diluted biotinylated anti-Ig (kappa chain), IgG1 or IgG2b were added to the samples and incubated at 4°C overnight. Plates were washed three times and then incubated with 100 μ l of 1/200 diluted goat anti-mouse streptavidin conjugate HRP (Sigma-Aldrich) for 30 min at 4°C. Samples were washed followed by addition of 100 μ l of 3,3',5,5'-tetramethylbenzidine chromogen solution. The plates were allowed to develop for 10 min, and then the reaction was stopped by adding 100 μ l stop solution. The OD was measured at 450 nm.

Histopathology

Eight randomly chosen mice from vehicle- and RTL-treated groups were perfused with 0.9% saline followed by cold 4% paraformaldehyde. Spinal cords were removed and post-fixed in 4% paraformaldehyde for 48 hrs. The spinal cords were dissected after fixation and embedded in paraffin before sectioning. The sections were stained with luxol fast blue/periodic acid-Schiff-hematoxylin to assess demyelination and inflammatory lesions, and analyzed by light microscopy. Inflammation (from 0-4) and demyelination (from 0-3) scores were determined as described by Chan(Chan et al., 2008). Inflammation was scored as: 0 = no inflammation, 1 = inflammatory cells only in leptomeninges and perivascular spaces, 2 = mild inflammatory infiltrate in spinal cord parenchyma, 3 = moderate inflammatory infiltrate in parenchyma, 4 = severe inflammatory infiltrate in parenchyma. Demyelination was scored as: 0 = no demyelination, 1 = mild demyelination, 2 = moderate demyelination, 3 = severe demyelination. Demyelination scores were determined independently by 2 investigators and reviewed for consensus if there were differences in scoring.

Immunohistochemistry

PBS-perfused lumbar spinal cord was fixed in 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C for at least 48h. The spinal cords were dissected from the spinal columns, cut into sections 1–2 mm length, re-fixed briefly in 10% Zn-buffered formalin,

dehydrated and embedded in paraffin blocks. Ten, 6µm thick sections were cut from paraffin blocks and mounted onto pre-cleaned microscope slides (Fisher Scientific, Pittsburgh, PA, USA). The sections were de-waxed and rehydrated sequentially with xylene (2 min), gradient ethanol (100%, 95%, 85%, 2 min each) and PBS (5 min), and then cooked (120°C) in antigen unmasking agent, Trilogy (Cell Marque, Hot Springs, AR, USA), for 10 min in a pressure steamer. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide in tap water for 5 min. The sections were incubated for 1h in working solution of Mouse Ig Blocking Reagent from the VECTOR M.O.M. immunodetection peroxidase kit (Vector Laboratories, Burlingame, CA, USA), and then incubated sequentially with primary antibody (SMI312 1:3000 diluted in M.O.M. diluent, 30min), M.O.M. biotinylated anti-Mouse IgG reagent (10 min), VECTASTAIN ABC reagent (5min), and DakoCytomation liquid DAB substrate (DakoCytomation, Carpinteria, CA, USA). The slides were dehydrated, and mounted with Cytoseal XYL mounting medium (Richard-Allan Scientific, Kalamazoo, MI, USA). Stained tissue sections were analyzed as described previously (Wang et al., 2006) by an investigator blinded to treatment status. The percentage of the spinal cord showing tissue damage was determined in the lumbar region of the cord. Damaged areas were measured using Image J 1.38x software. Measurements were also made of the total area (damaged and intact) of the dorsal columns and the lateral/ventral columns. Cumulative percent lesion areas were calculated for each region (dorsal column and lateral/ventral columns) and for the combined total damage from the two regions.

Statistical analysis

Statistical difference between vehicle and treatment groups was determined by the Mann-Whitney *U* test. Differences in cytokine levels, inflammation and axonal loss were evaluated by Student's *t* test. *p* values ≤0.05 were considered significant.

RESULTS

RTL551 reverses clinical signs of EAE induced by rhMOG/CFA/Ptx

At onset of clinical signs of actively-induced EAE (d10-13) mice were treated daily with 100µg RTL551 i.v. for 8 days. As shown in Figure 1, treatment with RTL551 was very effective in reducing the clinical severity of EAE and arresting disease progression throughout the observation period that lasted for 26 days post-immunization. The vehicle-treated mice showed a cumulative disease index (CDI) of 74.8±8.5, whereas the RTL-treated mice had a significantly reduced CDI of 47.8±12.5 ($p<0.01$, Fig. 1). The peak disease score of RTL551-treated mice was also reduced compared to vehicle-treated mice that developed severe EAE (5.2±0.35 vs. 4.5±0.7; $p<0.05$, Fig. 1). Four days after treatment completion, several mice in the RTL551-treated group relapsed. For this reason, all mice received booster injections for three more days prior to terminating the experiment on day 26 for ex vivo analyses. At the peak of disease (d14), 22% of mice were moribund and 11% of the mice died by the end of the experiment in the vehicle treated group. No mortality was observed in RTL551 treated mice.

Proliferation response and pro-inflammatory cytokine production to rhMOG are decreased in mice treated with RTL551

As shown in Fig. 2A, T cell proliferative responses of LN cells collected 26 days after EAE induction in vehicle-treated mice were much stronger to rhMOG (SI = 16X at 10µg/ml) than to hMOG-35-55 peptide (SI = ~3X), indicating multiple immunogenic T cell determinants in the rhMOG protein. Treatment with RTL551 significantly inhibited proliferative responses to both rhMOG protein and hMOG-35-55 peptide (Fig. 2A). The reduction in proliferation responses to rhMOG was paralleled by a decrease in secreted pro-inflammatory cytokines (TNF-α, IFN-γ and IL-17) by LN cultures from RTL551-treated vs. vehicle-treated mice (Fig 2B).

Ex vivo stimulation of splenocytes with rhMOG further supported the idea that RTL treatment suppresses disease-promoting pro-inflammatory cytokines early in the disease process. As shown in Figure 3, RTL551 treatment significantly reduced splenocyte secretion of TNF- α and IFN- γ on day 17 of EAE and TNF- α , IL-17 and MCP-1 levels on day 26 of EAE compared to vehicle-treated mice. No changes were noted in anti-inflammatory cytokines (ie. IL-4, IL-10 & IL-13) in RTL551-treated mice, in contrast to our prior study in RTL-treated SJL/J mice (Sinha et al., 2009).

RTL551 treatment does not decrease antibody levels to rhMOG protein in mice immunized with rhMOG

Clinical signs of EAE in C57BL/6 immunized with rhMOG are driven by a combination of T cell and antibody dependent effector mechanisms (Marta et al., 2005). We therefore investigated the serum antibody response to rhMOG in treated and control animals by ELISA to determine whether the therapeutic effects of RTL551-treatment was associated with a reduction in the rhMOG-specific antibody levels. Sera from animals immunized with synthetic peptides corresponding to either mMOG-35-55 or hMOG-35-55 peptides were used as antigen-specific controls in this study. Strikingly, treatment with RTL551 had no significant effect on high level Igk chain, IgG1 or IgG2b antibody responses to rhMOG (Figure 4; serum dilution 1:1000). To ensure this high antibody response to rhMOG did not obscure any selective effect on the response to the encephalitogenic peptide targeted by RTL551, we repeated the ELISA after sera were pre-absorbed with hMOG-35-55 peptide. Control experiments revealed that pre-adsorption of sera from animals immunized with either mMOG-35-55 or hMOG-35-55 peptide with the corresponding peptide completely abrogated detection of rhMOG-reactive antibodies by ELISA. In contrast, pre-adsorption of sera from rhMOG-immunized mice with hMOG-35-55 peptide had little or no effect on the ELISA signal to rhMOG, suggesting that antibody responses targeting this dominant encephalitogenic T epitope are not the major component of the rhMOG-specific antibody repertoire (Fig 4B). Collectively, our data indicate RTL551 treatment had no direct effect on antibody production by B cells, suggesting that the beneficial effects of RTL treatment in this disease model should be attributed to effects on T cell mediated inflammation in the CNS.

RTL551 reduces CNS pathology in mice immunized with rhMOG

Inflammation and demyelination were assessed in spinal cord tissue sections after luxol fast blue/periodic acid-Schiff-hematoxylin staining and scored as described in Methods. As shown in Fig. 5A and quantified in Fig. 5B, spinal cords of vehicle-treated mice showed intense subpial and perivascular inflammation and demyelination (as marked with arrows), whereas spinal cords of mice treated with RTL551 showed a significant decrease in infiltrating cells and demyelination.

The extent of axonal loss in spinal cords of rhMOG-immunized mice was determined by immunohistochemical staining with SMI312, an antibody cocktail for neurofilaments. As depicted in Fig. 6A and quantified in Fig. 6B, spinal cords of vehicle-treated mice had extensive loss of axons, particularly in the subpial region of white matter, corresponding with the presence of inflammation. On the contrary, axons in the RTL551-treated spinal cords were well preserved. The (mean) areas of axonal loss in the dorsal and lateral/ventral spinal cords of vehicle- vs. RTL551-treated mice were 41% and 58% vs. 4.3 and 3.2%, respectively ($p < 0.05$, Fig. 6B). Statistical analysis revealed that the extent of both axonal loss and neuroinflammation were significantly decreased in spinal cords from RTL551-treated mice (Fig 6B). It is noteworthy that axonal loss in rhMOG-immunized mice was much more extensive than in mMOG-35-55 peptide-immunized mice. Moreover, loss of axons correlated well with the clinical severity of the disease (assessed by cumulative disease index, CDI), which was nearly twice as high in vehicle-treated rhMOG-immunized mice as in mMOG-35-55 peptide-

immunized mice over the same observation period (CDIs = 74.8 ± 8.5 vs. 39.8 ± 6.0 , $p < 0.05$, respectively, data not shown). Furthermore, irrespective of the severity of axonal loss or clinical scores, RTL551 treatment successfully ameliorated clinical and histopathological signs of EAE in mice immunized with either rhMOG or mMOG-35-55 peptide.

DISCUSSION

It is now well established that rhMOG-induced EAE involves complementary cellular and humoral pathogenic components. On one hand, MOG-specific T cells initiate CNS inflammation and tissue damage, and on the other, MOG-specific antibody exacerbates this process, but only in the presence of a permissive blood-brain barrier. Our previous studies demonstrated that RTLs are very effective for treating T cell mediated EAE, but have not addressed the treatment of EAE involving a demyelinating antibody component that occurs in a subset of subjects with MS. Therefore, we evaluated the therapeutic effects of RTL551, specific for T cells reactive to mMOG-35-55 peptide, on EAE induced with rhMOG in C57BL/6 mice. The results clearly demonstrate the ability of RTL551 to reverse clinical paralysis, reduce T cell activation and block CNS inflammation, demyelination and axonal damage without suppressing the anti-MOG antibody response.

RTLs appear to modify T cell behavior as a partial agonist, signaling directly through the TCR to induce a CD3 ζ p23/p21 ratio shift, ZAP-70 phosphorylation, calcium mobilization, NFAT activation, transient IL-2 production (Wang et al., 2003) and constitutive secretion of IL-10 (Burrows et al., 2001). Thus, RTLs trigger specific downstream signaling events that deplete intracellular calcium stores without fully activating T cells. The resulting Ag-specific activation of the transcription factor, NFAT, uncoupled from the activation of NF κ B or ERKs constitutes a unique downstream activation pattern of T cells that modulates proliferation and cytokine production *in vitro* and may account for the inhibitory RTL effects on encephalitogenic CD4⁺ T cells *in vivo*.

The persistence of relatively high anti-MOG antibody levels in RTL-treated mice indicated that RTL treatment did not have any direct effect on antibody production by B cells. Rather, RTL mediated treatment of rhMOG induced EAE might be attributed to prevention of their entry into the CNS due to amelioration of early inflammatory responses. Perhaps a major effect of RTL551 therapy that could limit free access of anti-MOG antibody to the CNS is repair of the blood-brain barrier. We demonstrated previously that successful therapy of mMOG-35-55-induced EAE in C57BL/6 mice produced a 99% reduction in the expression of ICAM and VCAM by activated brain endothelial cells and disappearance of inflammatory MOG-reactive cells from spinal cord tissue (Sinha et al., 2007). Assuming similar blocking effects on vascular permeability for plasma proteins, RTL551 therapy may limit access of anti-MOG antibodies to levels insufficient to drive disease progression in the CNS, including the development of chronic deficits due to axonal pathology. More generally, our results imply that T cell-mediated inflammation and associated blood-brain barrier dysfunction are the central contributors to EAE pathogenesis, and that successful regulation of these key players restricts potential damage by demyelinating antibodies.

An important question arises as to how RTL551 might regulate T cell responses induced after immunization of C57BL/6 mice with the rhMOG protein. RTL551 is comprised of a truncated 2-domain I-A^b class II molecule with covalently tethered mMOG-35-55 peptide. On the other hand, rhMOG contains the hMOG-35-55 sequence (that differs from mMOG by a single S42P substitution) as well as other potentially encephalitogenic determinants that lie outside this region. We have demonstrated that immunization of HLA-DR2 mice with either mMOG-35-55 or hMOG-35-55 peptide can induce T cells that cross-react with the other sequence (Chou et al., 2004), and such cross-reactivity likely explains the weak encephalitogenic activity of

hMOG-35-55 peptide in C57BL/6 mice (peak EAE scores of ~2.0, HO unpublished observation). Crucial to this discussion, RTL551 can successfully treat EAE induced with either hMOG-35-55 peptide or mMOG-35-55 peptide (HO unpublished observation). Therapeutic activity directed against the hMOG-35-55 determinant as well as bystander T cells (Sinha et al., 2009) reactive against other rhMOG determinants could explain how RTL551 might regulate multiple T cell specificities that likely contribute to rhMOG-induced EAE. This potent regulatory effect on the T cell component of EAE could then prevent anti-MOG antibody enhancement of disease as discussed above without direct effects on antibody responses or MOG-reactive B cells.

The results of our study lend support for the use of RTL therapy for treatment of MS subjects whose disease includes inflammatory T cells as well as those with an additional antibody component. Thus, RTL1000 that is comprised of HLA-DR2 (DRB1*1501) linked to hMOG-35-55 peptide, conceivably could regulate pathogenic hMOG-35-55 reactive T cells and limit additional antibody-driven CNS damage, including the demyelinating effects of anti-MOG antibodies. A Phase I clinical trial using escalating doses of RTL1000 has been completed successfully in subjects with MS, with additional trials to follow. Information from our current study presented above clearly expands the possible therapeutic potential of RTL therapy to include antibody-mediated damage to CNS myelin and axons.

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Group	Incidence	Onset	Peak	CDI
Vehicle	18/18	11.3±0.9	5.2±0.4	74.8±8.5
RTL551	17/17	11.1±0.7	4.5±0.7 ^a	47.8±12.5 ^b

^avehicle vs. RTL551, p<0.05; ^bvehicle vs. RTL551, p<0.01

Figure 1. Intravenous administration of RTL551 treats rhMOG induced EAE in C57BL/6 mice
C57BL/6 male mice were immunized with rhMOG/CFA/Ptx. At disease onset when clinical scores were ≥ 2.0 , mice were treated daily with vehicle or 100 μ g of RTL551 for 8 days. To graphically synchronize effects of RTL treatment on disease course, the onset was normalized to Day 10 in the Figure. Mice also received booster injections for 3 days beginning on Day 22 as indicated in the Figure and were scored as outlined in *Materials and Methods*. Black arrows indicate i.v. injection of RTL551 and grey arrows indicate s.c. injection. Data presented are the mean of three experiments involving a total of 23-24 mice per group from which 6 mice were sampled on Day 17 and the remaining mice sampled on Day 26 for ex vivo analyses. Significant differences between control and treated groups were determined using the Mann Whitney test (^a, p < 0.05).

Figure 2. RTL551 treatment suppresses proliferative response and inflammatory cytokine production from lymph node cells of mice with rhMOG induced EAE

Lymph node cells from vehicle-treated mice showed a 16X proliferative response to rhMOG (at the higher protein concentration), and this response was significantly downregulated in RTL551-treated mice. Lymph nodes were isolated from vehicle- and RTL-treated groups of mice 26 days post immunization. The cells from four representative mice per group were pooled and T cell responses were measured by proliferation to rhMOG and hMOG-35-55 peptide after 72-h incubation, the last 18h in presence of [3 H] thymidine (A). Data are presented as stimulation index (SI) relative to media alone controls and are representative of three separate experiments. Along with reduced proliferation, *ex vivo* cultured cells from RTL551-treated mice had significantly reduced production of inflammatory cytokines, TNF- α , IFN- γ and IL-17 (B). LN cells from each group were cultured *in vitro* with 10 μ g/ml of rhMOG, and supernatants were harvested after 48 hr and assayed for cytokine production using the Bio-Plex cytokine assay kit. Data are presented as the mean $\pm$ SD of three replicate cultures from pooled cells, and are representative of three experiments. Significant differences between control and treated groups are indicated (* = $p < 0.05$, Student's *t* test).

Figure 3. Pro-inflammatory response in the spleen is attenuated by RTL treatment early in rhMOG induced EAE

Mice were sacrificed 17 or 26 days after immunization and splenocytes from each group were cultured *ex vivo* with 10 μ g/ml of rhMOG. Supernatants were harvested after 48 hr and assayed for cytokine production using Bio-Plex cytokine assay kit. Data are presented as the mean $\pm$ SD of three replicate cultures from pooled cells, and are representative of three experiments. Significant differences between control and treated groups are indicated (* = $p < 0.05$, Student's *t* test).

Figure 4. RTL551 treatment does not decrease antibody responses in mice with rhMOG-induced EAE

Serum samples from rhMOG immunized mice contains high titers of antibodies to rhMOG (A), which are not removed by adsorption with hMOG-35-55 peptide (B), suggesting that they might be directed to other linear or conformational determinants. Serum samples were obtained 26 days post immunization and half of the samples were adsorbed with hMOG-35-55 prior to running ELISAs on plates coated with rhMOG. Serum samples from mMOG-35-55 immunized mice treated or not with RTL551 are included as controls. Data are presented as the mean $\pm$ SD of ELISA results obtained from sera of 17-18 mice per group, and are representative of three experiments.

Figure 5. RTL551 reduces CNS pathology in mice with rhMOG-induced EAE

Fixed, paraffin-embedded spinal cord sections from vehicle- or RTL551-treated C57BL/6 mice taken 26 days after immunization with rhMOG/CFA/Ptx showed dense mononuclear infiltration (arrows) and loss of myelin staining (luxol fast blue) in regions adjacent to the mononuclear infiltrate (A). Spinal cords from RTL-treated mice showed lack of inflammatory cells with no apparent loss of myelin. Magnification: $\times 4$. Mean inflammation and demyelination scores of 8 cords per group are represented in (B). Significant differences between control and treated groups are indicated (* = $p < 0.05$, Student's t test).

Figure 6. RTL551 prevents axonal loss in spinal cords of mice immunized with rhMOG

Representative phosphorylated neurofilament staining of axons in lumbar spinal cord sections from EAE mice treated with vehicle (left panel) or RTL551 (right panel) at disease onset (A). Tissue sections were dissected from PBS perfused mice (8 randomly chosen mice from each group), fixed and stained with SMI312, an antibody cocktail for neurofilaments (brown) (A). Morphometric analysis of the development of axonal loss in EAE mice treated with vehicle or RTL551 (B). Digitally acquired images were analyzed with Image J software. Areas with loss of phosphorylated neurofilament staining of axons were circled by hand and the percentage of axonal loss area was calculated by dividing total axonal loss areas by the total area of dorsal or lateral/ventral white matter (B). Data represent mean $\pm$ SD. Significant differences between RTL-treated vs. vehicle control groups are indicated (* = $p < 0.05$, Student's t test).