Recombinant TCR Ligand Induces Tolerance to Myelin Oligodendrocyte Glycoprotein 35-55 Peptide and Reverses Clinical and Histological Signs of Chronic Experimental Autoimmune Encephalomyelitis in HLA-DR2 Transgenic Mice

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Recombinant TCR Ligand Induces Tolerance to Myelin Oligodendrocyte Glycoprotein 35-55 Peptide and Reverses Clinical and Histological Signs of Chronic Experimental Autoimmune Encephalomyelitis in HLA-DR2 Transgenic Mice


In a previous study, we demonstrated that myelin oligodendrocyte glycoprotein (MOG)-35-55 peptide could induce severe chronic experimental autoimmune encephalomyelitis (EAE) in HLA-DR2 transgenic mice lacking all mouse MHC class II genes. We used this model to evaluate clinical efficacy and mechanism of action of a novel recombinant TCR ligand (RTL) comprised of the α1 and β1 domains of DR2 (DRB1*1501) covalently linked to the encephalitogenic MOG-35-55 peptide (VG312). We found that the MOG/DR2 VG312 RTL could induce long-term tolerance to MOG-35-55 peptide and reverse clinical and histological signs of EAE in a dose- and peptide-dependent manner. Some mice treated with lower doses of VG312 relapsed after cessation of daily treatment, but the mice could be successfully re-treated with a higher dose of VG312. Treatment with VG312 strongly reduced secretion of Th1 cytokines (TNF-α and IFN-γ) produced in response to MOG-35-55 peptide, and to a lesser degree purified protein derivative and Con A, but had no inhibitory effect on serum Ab levels to MOG-35-55 peptide. Abs specific for both the peptide and MHC moieties of the RTLs were also present after treatment with EAE, but these Abs had only a minor enhancing effect on T cell activation in vitro. These data demonstrate the powerful tolerance-inducing therapeutic effects of VG312 on MOG peptide-induced EAE in transgenic DR2 mice and support the potential of this approach to inhibit myelin Ag-specific responses in multiple sclerosis patients. The Journal of Immunology, 2003, 171: 127–133.

Transgenic (Tg) mice expressing HLA class II molecules have a precise testing ground for screening disease-associated T cell determinants (1, 2). HLA-DR4-restricted T cell responses, and in some cases clinical signs of autoimmune disease, were demonstrated after immunization with type II collagen (3–5), human cartilage Ag gp39 (6), glutamic acid decarboxylase 65 (7, 8), insulin (9), and myelin oligodendrocyte glycoprotein (MOG)-91-108 peptide (10). HLA-DR2+ mice have also developed that were susceptible to experimental autoimmune encephalomyelitis (EAE) induced by myelin basic protein (MBP)-85-99 peptide (11), proteolipid protein-95-116 peptide (12), and MOG-35-55 peptide (A. A. Vandenbark, C. Rich, A. Zamora, H. Jacobsen, H. Offner, R. Jones, and L. Fugger, manuscript in preparation). Of many myelin determinants that might serve as target T cell epitopes in multiple sclerosis (MS), the MOG-35-55 peptide is of particular interest. MOG appears to be recognized frequently by T cells from MS patients (13, 14), and the MOG-35-55 peptide has been shown to be highly encephalitogenic in rats and mice on different MHC class II backgrounds (14–17) and in Rhesus monkeys (18, 19).

One approach to regulating Ag-specific T cell responses to encephalitogenic peptides is to induce nonresponsiveness using TCR ligands containing extracellular domains of class II MHC molecules linked to specific peptide targets. Several such constructs have been developed that involve natural or recombinant α1β2 or β1β2 MHC class II domains in association with various encephalitogenic or other pathogenic peptides that were either linked covalently or bound noncovalently (20–24). These molecular complexes bind not only to the TCR but also to the CD4 molecule on the T cell surface through the β2 MHC domain (25) and were found to inhibit T cell activation and prevent EAE in rodents (22, 26, 27). Our design includes only the minimal TCR interface, which involves only the α1 and β1 MHC domains without CD4 binding covalently linked to peptide (28). These constructs can prevent and treat MBP-induced EAE in Lewis rats (29, 30) and inhibit activation and induce IL-10 secretion in human DR2-restricted T cell clones specific for MBP-85-99 or BCR-ABL b3a2 peptide (CABL) peptides (31, 32). To further evaluate the inhibitory activity and mechanism of the effects of recombinant TCR ligand (RTL) on encephalitogenic T cells in vivo, we designed a MOG-35-55/DR2 construct (VG312) for testing in DR2 mice undergoing MOG peptide-induced EAE. Our results demonstrate potent
inhibitory activity resulting in immunological tolerance to the encephalitogenic MOG-35-55 peptide and reversal of clinical and histological signs of EAE.

Materials and Methods

Mice

HLA-DR2 Tg mice bearing chimeric MHC class II molecules were developed as previously described (33). The peptide-binding domain of MHC class II is encoded by human sequences, whereas the membrane-proximal portion including the CD4-binding domain is encoded by mouse sequences (DRα1*0101: I-Eα, and DRβ1*1501: I-Eβ). The DRα1*0101: I-Eα construct was kindly provided by Dr. D. M. Zaller (Merck Research Laboratories, Rahway, NJ). The DRβ1*1501: I-Eβ construct was made essentially as described by Woods et al. (33) with the following changes: the pACYC184 vector containing the DRB1*0401 exons 1 and 2 and the Eβ' exons 3–6 were partially digested with BamHI and treated with Klenow polymerase to remove a BamHI site in the vector. Subsequently, DRB1*1501 exon 2 was cloned into pACYC184, which had been predigested with BamHI and EcoRI to remove DRB1*0401 exon 2. Tg mice were generated by microinjecting the chimeric α- and β-chain constructs into fertilized eggs from (DBA/2 × C57BL/6)F1 matings. Viable embryos were transferred into pseudo-pregnant females for development to term. Tg offspring were backcrossed twice to the MHC class II knockout mouse, MHCβ–Δ1 (34).

Antigens

Mouse MOG-35-55 peptide (MEVGWYRSPFSRVHLYRNGK) was synthesized using solid-phase techniques and purified by HPLC at the Beckman Institute, Stanford University (Palo Alto, CA). Purified protein derivative (PPD) was purchased from the Statens Serum Institute (Copenhagen, Denmark). Con A was purchased from Sigma-Aldrich (St. Louis, MO).

Construction ofRTLs

Single chain human RTLs of ~200 aa residues derived from HLA-DR2b were designed using the same principles as for rat RTLs (28) and have been produced in Escherichia coli with and without amino-terminal extensions containing antigenic peptides (32). Similar to the rat constructs, human RTLs exhibited a cooperative two-state thermal unfolding transition, and DR2-derived RTLs with a covalently linked MBP-85-99 peptide or mouse MOG-35-55 peptide showed increased stability to thermal unfolding relative to the empty DR2-derived RTLs.

Induction of active EAE and treatment withRTLs

Tg HLA-DR2 male and female mice between 8 and 12 wk of age were immunized s.c. as described (17) at four sites on the flanks with 0.2 ml of an emulsion comprised of 200 µg of mouse MOG-35-55 peptide in CFA containing 400 µg of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). In addition, mice were given pertussis toxin (Ptx) from List Biological Laboratories (Campbell, CA) on days 0 and 2 postimmunization (25 and 67 ng per mouse, respectively). Mice were treated i.v. daily for 8 days, beginning 2–4 days after onset of clinical signs, with 100 µl of VG312 (MOG-35-55 peptide/DR2), VG303 (MBP-85-99 peptide/DR2), VG311 (CABL peptide/DR2), or vehicle (Tris, pH 8.5) containing 0–100 µg of various RTL proteins. Actively immunized mice were assessed daily for clinical signs of EAE according to the following scale: 0 = normal; 1 = limp tail or mild hind limb weakness; 2 = limp tail and moderate hind limb weakness or mild ataxia; 3 = limp tail and moderately severe hind limb weakness; 4 = limp tail and severe hind limb weakness or moderate ataxia; 5 = limp tail and paraplegia with no more than moderate forelimb weakness; and 6 = limp tail and paraplegia with severe forelimb weakness or severe ataxia or moribund condition. The average daily score was determined for each group of mice by summing the individual scores and dividing by the number of mice in the group. Mice that occasionally die from severe EAE are given a score of 6 and are included in the daily score. The cumulative disease index (CDI) was determined by summing the daily clinical scores for each mouse. The mean peak scores, the average daily scores, and the CDI scores ± SD were calculated for the control and experimental groups and were evaluated for statistical differences using the Kruskal-Wallis test for multivariable analyses of nonparametric comparisons.

Proliferation assay

Draining lymph node (LN) cells from HLA-DR2 Tg mice were recovered at the indicated time points following immunization and processed into single-cell suspensions. T cell proliferation responses were assessed by plating 4 × 10^6 cells per well in a 96-well flat-bottom tissue culture plate in stimulation medium alone (control) or in the presence of Ags. Cultures were incubated for 72 h at 37°C in 7% CO2. Wells were pulsed for the final 18 h with 0.5 µCi per well [3H]thymidine (Amersham, Arlington Heights, IL). The cells were harvested onto glass fiber filters, and [3H]thymidine uptake was measured using a liquid scintillation counter (1205 Betaplate; Wallac, Turku, Finland). Mean cpm ± SD were calculated for triplicate wells.

Cytometric bead array

TNF-α, IFN-γ, IL-2, IL-4, and IL-5 were simultaneously detected in supernatants from the indicated cell source using the mouse Th1/Th2 cytokine cytometric bead array kit from BD Biosciences (San Jose, CA). A total of 50 µl of supernatant, 50 µl of a mixture of capture beads, and 50 µl of Th1/Th2 PE detection reagent were combined and incubated for 2 h at room temperature in the dark. The samples were washed, resuspended in ~200 µl of wash buffer, and analyzed by flow cytometry (FACSCalibur; BD Biosciences). Standard curves were generated for each cytokine using a mouse standard curve for each cytokine. The concentration of cytokine in the cell supernatant was measured using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA) and quantified using a standard curve.

Assessment of Ab response to peptide Ags by indirect ELISA

Sera were isolated from individual animals, pooled, and frozen at ~80°C. ELISA plates (Nunc Maxisorp; Nalge Nunc International, Roskilde, Denmark) were coated with 10 µg/ml mouse MOG-35-55 peptide in 100 µl of PBS overnight at 4°C. Plates were then washed with 1× PBS/0.05% Tween 20 and blocked with 200 µl of 1× PBS/5%BSA/0.05% Tween 20 for 2 h at 37°C. Plates were washed, and plate-bound Ab was detected with anti-mouse IgG H and L chain conjugated to streptavidin-HRP (Bethyl Laboratories, Montgomery, TX). Plates were developed for ~10 min in the dark using 3,3',5,5'-tetramethylbenzidine as a substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The reaction was stopped using tetramethylbenzidine stop solution, and the plates were immediately read at 490 nm on a kinetic microplate reader (Molecular Devices).

Histopathology

The intact spinal cord was removed from mice euthanized 24–38 days after induction of EAE and fixed in 10% phosphate-buffered formalin. The spinal cords were dissected after fixation and embedded in paraffin before sectioning. The sections were stained with either Luxol fast blue/periodic acid-Schiff’s reagent or H&E and analyzed by light microscopy. Semi-quantitative analysis of inflammation and demyelination was determined by examining at least 10 sections from each mouse.

Results

Peptide- and dose-dependent inhibition of MOG-induced EAE in 1501 mice

As recently described (A. A. Vandenbark, C. Rich, A. Zamora, H. Jacobsen, H. Offner, R. Jones, and L. Fugger, manuscript in preparation), MOG-35-55-induced EAE in Tg DR2 (DRB1*1501) mice is characterized as a moderately severe chronic disease affecting 100% of the mice with ascending paralysis marked by inflammatory, demyelinating CNS lesions. EAE was induced with MOG-35-55 peptide/CFA on day 0 plus Ptx on days 0 and 2. The initial signs of disease could be observed beginning ~10 days after induction. To evaluate the clinical potential of human DR2-restricted RTLs, we treated Tg DR2 mice with MOG-induced EAE 2–4 days after onset of clinical signs with VG312 (MOG-35-55/DR2) or control constructs containing different DR2-restricted peptides, VG303 (MBP-85-99/DR2) or VG311 (CABL-peptide/DR2) (31, 32). Each construct was produced and characterized in an identical manner as described. The treatment regimen involved...
daily i.v. injections of 100, 33, or 10 μg of the indicated construct in 100 μl of Tris buffer, or Tris buffer alone (vehicle) for 8 consecutive days.

As shown in Fig. 1, treatment with VG312 at doses of 100 or 33 μg/injection rapidly reversed established clinical signs of EAE (score ~2.5) to an average daily score of <0.5 U by the end of the
8-day treatment period (combined data from four separate experiments). This low degree of disability was maintained without further RTL injections over the remainder of the observation period, which in one experiment lasted for 5 wk after treatment was stopped. In contrast to the reversal of EAE mediated by the higher doses of VG312, control groups receiving vehicle or 100 μg/injection VG303 (MBP-85-99/DR2) or VG311 (CABL-peptide/DR2) developed moderately severe chronic EAE (score of 4). The clinical improvement observed in VG312-treated mice was reflected by a marked reduction of inflammatory lesions and demyelination in spinal cord tissue (Fig. 2).

As shown in Fig. 3, treatment of EAE in Tg DR2 mice with VG312 was dose dependent. Similar to the 100- and 33-μg doses of VG312, the 10-μg dose of VG312 also reduced the clinical score to baseline after the first course of eight injections. However, unlike mice receiving the higher doses of VG312 that experienced long-lasting clinical benefit, two of the three mice in the 10-μg group in Fig. 3 and three of the four mice treated with 10 μg of VG312 in a repeat experiment relapsed after treatment was stopped. One relapsed mouse from this group was re-treated with three injections of 33 μg VG312, resulting in clinical improvement from a score of 4 to a score of 2 (Fig. 4). This mouse experienced signs of hypersensitivity (see below), and therefore was given antihistamine at the same time as the second course of VG312. As an additional control, Tg DR2 mice with EAE were treated with 10 or 3.3 μg of free MOG-35-55 peptide, which represents the same molar equivalence of MOG-35-55 peptide found in the 100- and 33-μg dose of VG312. Free peptide at both concentrations reduced clinical scores of EAE to baseline during the first course of eight treatments, but in both cases some of the mice experienced relapses within 2 days after the course of treatment was completed (Fig. 5).

Treatment of EAE with VG312 inhibits MOG-35-55-specific T cell proliferation and secretion of inflammatory cytokines

To evaluate the effects of VG312 treatment, LN cells were collected and tested for T cell responses to the encephalitogenic MOG-35-55 peptide. As shown in Fig. 6, the proliferation responses of LN cells from VG312-treated mice stimulated ex vivo with three concentrations of MOG-35-55 peptide were profoundly (>90%) inhibited compared with responses of LN cells from vehicle-treated mice. In contrast, treatment with VG312 produced moderate inhibition of responses to PPD (28%) and Con A (34%) (Fig. 6). Surprisingly, T cell proliferation responses appeared to be enhanced at the 10- and 2-μg concentrations of MOG-35-55 peptide in Tg DR2 mice treated with VG311 compared with those treated with vehicle (Fig. 6). Of importance mechanistically, addition of IL-2 to unresponsive T cell cultures did not enhance responses to MOG-35-55 peptide or PPD beyond the effects of IL-2 alone (data not shown), indicating that the lack of response to MOG-35-55 peptide was not caused by functional anergy. The strongly reduced T cell proliferation responses to MOG-35-55 peptide were reflected by a concomitant reduction in secretion of TNF-α and particularly IFN-γ (Fig. 6), as well as IL-2 (data not shown), as measured by cytokine bead analysis. In addition, treatment with VG312 but not VG311 reduced consistently, but to a lesser degree, the secretion of TNF-α, IFN-γ (Fig. 6), and IL-2 (data not shown) by LN cells stimulated with PPD and Con A, indicating bystander suppression. Other cytokines measured in this...
assay, including IL-4 and IL-5 as well as IL-10 measured by ELISA, were not detected at significant levels in either the vehicle-treated or VG312-treated mice in response to MOG-35-55 peptide, PPD, or Con A (data not shown).

**Immunogenicity of VG312 components after injection in vivo**

Although the sequence of VG312 is based on the germline sequences of human HLA-DR2 (DRA:DRB1*1501) and mouse (self) MOG-35-55 peptide, alterations required to generate a single chain RTL construct (e.g., linker regions and amino acid substitutions) might be immunogenic in Tg DR2 mice undergoing treatment for EAE (32). To evaluate immunogenic components of VG312, LN cells and Abs were tested for recognition of VG312, VG311 (with a different bound peptide), VG302 (empty RTL without peptide or linker), and MOG-35-55 peptide. As shown in Fig. 6, LN cells from VG312-treated Tg DR2 mice developed significant proliferation and cytokine responses to VG312, thus indicating that this construct was immunogenic in vivo.

The pattern of serum Ab responses to RTL components revealed a substantial baseline recognition of the empty DR2 moiety (VG302) and VG311 in vehicle-treated mice that was strongly enhanced in VG312 and VG311-treated Tg DR2 mice with EAE (Fig. 7). However, Abs specific for the encephalitogenic MOG-35-55 peptide were also present in VG312- and vehicle-treated mice (Fig. 7). The combination of these components resulted in a vigorous Ab response to the VG312 molecule in VG312- and vehicle-treated mice. Similarly, a vigorous Ab response was observed to VG311 in VG311-treated mice, with Ab recognition of the CABL peptide being present only in antisera from the VG311-treated mice (data not shown). To further evaluate the possible neutralizing effects of Abs induced in the RTL-treated mice, antisera from various groups of donors were incubated with splenocytes from MOG-35-55-immunized mice in the presence of Con A, PPD, or various dilutions of MOG-35-55 peptide. We found that antisera from VG312-treated mice did not have any detectable neutralizing effect on T cell responses to MOG-35-55 peptide or control Ags, and may have had a slight enhancing effect compared with antisera from VG303- or vehicle-treated mice (data not shown). No activating effects of these antisera were noted in the absence of Ag or mitogen stimulation.

**Discussion**

Our results demonstrate the potent activity of a minimal human TCR ligand to reverse clinical signs of EAE and induce long-term T cell tolerance against the encephalitogenic, DR2-restricted, MOG-35-55 peptide in Tg mice uniquely expressing this MS-associated HLA-DR2 allele. Immunization of the Tg DR2 mice with MOG-35-55 peptide induced strong T cell responses, perivascular spinal cord lesions with demyelination, and severe chronic signs of EAE, as well as anti-MOG Abs that were apparently not involved in either disease or tolerance induction. Treatment of the Tg DR2 mice after onset of clinical EAE with an 8-day course of daily i.v. injections of 100 or 33 μg of VG312 reversed disease progression to baseline levels, prevented or reversed formation of inflammatory lesions and demyelination, and maintained reduced clinical activity even after cessation of further injections. Treatment with a lower dose of 10 μg of VG312 or molar equivalent doses of free peptide corresponding to the 100- or 33-μg dose of VG312 also...
reduced clinical signs after the course of eight injections, but clinical benefit was not maintained, and most of these mice (i.e., five of seven mice treated with the 10-μg dose of VG312) had clinical relapses and disease progression. Treatment with control DR2 RTLs linked to other peptides (e.g., MBP-87-99 or CABL-peptide) did not inhibit EAE or affect T cell responses to MOG-35-55 peptide, demonstrating peptide specificity. Finally, re-treatment of relapsed mice with a higher dose of VG312 again produced clinical benefit, although mild signs of hypersensitivity were detected that were easily treated with antihistamines.

The treatment regime with VG312 used in our study profoundly inhibited T cell responses to the encephalitogenic MOG-35-55 peptide, including proliferation and secretion of the proinflammatory cytokines TNF-α, IFN-γ, and IL-2. The Th2 cytokines IL-4, IL-5, and IL-10 were not detected in mice developing EAE and were not induced after treatment with VG312. In a previous study using VG303 and VG311 (31), in vitro treatment of human DR2-restricted T cell clones specific for the respective cognate peptides MBP-85-99 and CABL produced partial agonist signaling through the TCR, resulting in default induction of IL-10. Our current study of DR2 mice did not detect any induction of IL-10 in vivo using VG312, thus indicating possible differences in signaling pathways in mouse vs human T cells. However, T cell proliferation and inflammatory cytokine responses to PPD and Con A were moderately to strongly reduced in VG312- vs vehicle-treated mice, indicating possible bystander suppression exerted by VG312. However, it remains
to be seen whether the inhibitory mechanism affects bystander T cell responses to other encephalitogenic determinants.

Attempts to overcome possible anergy and thus rescue MOG-35-55-specific T cells with IL-2 did not enhance T cell responses to MOG-35-55 peptide. Yet, nonresponsiveness to the MOG-35-55 peptide persisted for up to 5 wk after cessation of treatment, strongly suggesting either potent suppression or a deleting mechanism. These results are not inconsistent with a mechanism of anergy leading to eventual late apoptosis proposed by Appel, Wucherpfennig, and colleagues (35) to explain inhibition of human T cells with four-domain DR2/MBP-85-99 dimers.

The DR2 RTL constructs were found to be immunogenic in the treated DR2 mice, inducing both T cell and Ab responses, primarily to the covalently linked highly antigenic MOG-35-55 peptide. However, some T cell and Ab reactivity was directed against the DR2 moiety itself, presumably to minor sequence changes required for correct folding of the single chain construct or to linker sequences attaching the MOG-35-55 peptide to the DR2 moiety or joining the DRAα and the DRBβ domains (32). These T cell and Ab responses did not appear to affect the therapeutic activity of VG312 on EAE. However, antisera from VG312-treated mice appeared to moderately enhance responses of MOG-specific T cells in vitro compared with antisera from VG303- or vehicle-treated mice, suggesting some Ab recognition of the MOG peptide/DR2 complex that facilitated activation.

In conclusion, our study demonstrates for the first time the potent therapeutic effects of a rationally designed minimal TCR ligand in a humanized model of EAE. These results strongly support the application of this novel class of peptide/MHC class II constructs, and specifically the VG312 construct directed against the DR2-restricted MOG-35-55 peptide for treatment of T cell-mediated autoimmune diseases such as MS.

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References