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A Switch in Pathogenic Mechanism in Myelin Oligodendrocyte Glycoprotein-Induced EAE in GILT-Free Mice

Cheryl M. Bergman[*], **Cecilia B. Marta**[†], **Maja Maric**[‡], **Steven E. Pfeiffer**[§], **Peter Cresswell**[¶], and **Nancy H. Ruddle**[*,¶]

[*]Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06520-8034

[‡]DHHS/NIH/NIAID/DEA/SRP, Bethesda, MD 20892-7616

[¶]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520-8011

Abstract

Gamma interferon-inducible lysosomal thiol reductase (GILT) is an enzyme located in the Lamp-2 positive compartments of antigen presenting cells. GILT$^{-/-}$ mice are phenotypically normal but their T cells exhibit reduced proliferation to several exogenously administered antigens that include cysteine residues and disulfide bonds. We undertook the present studies to determine if GILT$^{-/-}$ mice would process exogenously administered myelin oligodendrocyte glycoprotein (MOG), which contains disulfide bonds, to generate experimental autoimmune encephalomyelitis (EAE) to the endogenous protein. One possibility was that MOG$_{35–55}$ peptide would induce EAE, but that MOG protein would not. GILT$^{-/-}$ mice were relatively resistant to MOG$_{35–55}$–induced EAE but slightly more susceptible to rat MOG protein-induced EAE than wild-type (WT) mice. Even though MOG$_{35–55}$ was immunogenic in GILT$^{-/-}$ mice, GILT antigen presenting cells could not generate MOG$_{35–55}$ from MOG protein in vitro, suggesting that the endogenous MOG protein was not processed to the MOG$_{35–55}$ peptide in vivo. Immunization of GILT$^{-/-}$ mice with rat MOG protein resulted in a switch in pathogenic mechanism from that seen in WT mice; the CNS infiltrate included large numbers of plasma cells; GILT$^{-/-}$ T cells proliferated to peptides other than MOG$_{35–55}$. In contrast to WT rat MOG-immunized mice, rat MOG-immunized GILT$^{-/-}$ mice generated antibodies that transferred EAE to MOG$_{35–55}$ primed GILT$^{-/-}$ mice and these antibodies bound to oligodendrocytes (OLs). These studies, demonstrating the key role of a processing enzyme in autoimmunity, indicate that subtle phenotypic changes have profound influences on pathogenic mechanisms, and are directly applicable to the out-bred human population.

INTRODUCTION

The immune system loses its ability to distinguish self from non-self in autoimmune diseases. These diseases are multi-factorial with influences of genetics and environment.

Address correspondence and reprint requests to Dr. Nancy H. Ruddle, PhD, Yale University School of Medicine, P.O. 208034, New Haven, CT 06520-8034. Telephone: 203-785-3281, Fax: 203-785-7903, nancy.ruddle@yale.edu.
[§]Deceased
[†]Current Address: Sanofi-Aventis Bridgewater, NJ 08870

Making them more complex is the fact that an individual disease can exhibit identical clinical manifestations, though the pathogenic mechanisms may vary widely. Such is the case in multiple sclerosis (MS), an inflammatory demyelinating disease presenting with a variety of clinical manifestations that include relapsing-remitting, primary progressive and secondary progressive subtypes. Even when the clinical signs are similar, several different pathogenic mechanisms have been postulated. (1–4) These include: effector T cells and cytokines, antibodies or B cells, and/or oligodendrocyte dystrophy. In the latter case, inflammation has been postulated to be secondary to the intrinsic tissue damage. The challenge in devising treatment for any autoimmune disease is to identify the pathogenic mechanism in the individual patient in order to choose the optimal therapy. This becomes even more daunting when the clinical signs are similar, but the pathogenic mechanisms differ.

In our previous studies, we have used myelin oligodendrocyte glycoprotein (MOG) induction of experimental autoimmune encephalomyelitis (EAE) in the mouse as a model of MS. MOG is a trans-membrane protein expressed on the cell body and processes of oligodendrocytes (OLs) and on the outermost lamellae of the myelin sheath. It is a type I integral membrane protein with a single extracellular domain (ECD). MOG is normally glycosylated at asparagine 24 and can multimerize (5). $MOG_{35–55}$, the immunodominant peptide for C57BL/6 (B6) mice, lies at a dimer interface (5). Immunization of C57BL/6 mice with rodent MOG $peptide_{35–55}$ ($MOG_{35–55}$) results in EAE that is CD4 T cell-mediated, B cell-independent, and involves both TH1 and TH17 cells and their cytokines (6–8). We previously showed that immunization with the ECD of recombinant rat MOG protein (rratMOG) also induces a T cell-mediated, B cell-independent EAE. However, immunization with recombinant human MOG protein (rhuMOG), even though it elicits similar clinical signs, is a B cell-dependent disease (9). This is due to a difference in a particular amino acid residue, a proline, rather than a serine at position 42 in human MOG. Immunization of WT mice with the ECD of human MOG results in the generation of a pathogenic antibody that recognizes glycosylated MOG on the surface of OLs (10) and induces EAE in human MOG-primed B cell deficient mice. These data indicate that a minor switch in immunizing antigen can have a profound effect on the pathogenic mechanism in autoimmune disease, even in mice of the same genetic background.

Protein presentation to CD4 T cells requires processing to peptides that can bind to MHC class II and be recognized by the T cell receptor in that context. In the case of exogenously administered rratMOG to WT mice, this results in class II-associated peptides that include $MOG_{35–55}$, the predominant encephalitogenic epitope in C57BL/6 mice (11, 12). Gamma interferon-inducible lysosomal thiol reductase (GILT) is located in the Class II-positive, Lamp-2 positive compartments of APCs (13–16). Mice deficient in GILT ($GILT^{-/-}$ mice) are phenotypically normal, but their T cells exhibit reduced proliferation to hen egg lysozyme (HEL), RNAse A, and human immunoglobulin G, all proteins with cysteine residues and disulfide bonds (16). Such is not the case with α-casein, which has no disulfide bonds. In the case of HEL, presentation of one of the epitopes is severely reduced, while other cysteine containing epitopes, are not affected (16). GILT is therefore essential for facilitating processing and presentation of some, but not all, peptides in exogenously administered protein antigens containing disulfide bonds. In rat and mouse MOG, cysteine

residues are located in the extracellular domain at positions 24 and 98 and form an internal disulfide bond that includes the predominant $MOG_{35–55}$ epitope (Fig. 1). Additional cysteines are also located in the intracellular region at positions 177 and 198. These features suggested a potential role for GILT in the CD4 T cell response to MOG.

Here, we evaluated the role of GILT in MOG-induced EAE, an autoimmune disease that is induced by injection of the exogenous antigen but also depends on presentation of the endogenous antigen. We hypothesized that $GILT^{-/-}$ mice would be protected from EAE induced by rratMOG because their APCs would be reduction-deficient and therefore unable to generate the encephalitogenic peptide $MOG_{35–55}$ from the immunizing protein. However, they might be susceptible to EAE when immunized with $MOG_{35–55}$ that would not require processing, although the possibility remained that the endogenous MOG would need to be processed and these mice might be resistant as well. Peptide-immunized $GILT^{-/-}$ mice were relatively resistant to EAE, though their T cells recognized the peptide immunogen. On the other hand, rat MOG protein-immunized $GILT^{-/-}$ mice developed EAE. These mice demonstrated a switch from the usual effector T cell-mediated pathogenic mechanism generated by rratMOG in WT mice, to one that was B cell-dependent, with pathogenic antibodies that recognized MOG on the surface of OLs. Furthermore, T cells isolated from rratMOG-immunized $GILT^{-/-}$ mice did not recognize the usually dominant epitope, $MOG_{35–55}$, but underwent a switch in epitope recognition to peptides that included those not dependent on breaking the disulfide bond. These studies support the concept that subtle differences in antigen processing can result in different pathogenic mechanisms and emphasize the importance of recognition of both the immunizing and endogenous antigens in autoimmunity.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) female mice between the ages of 6 and 10 weeks were obtained from The Jackson Laboratories, Bar Harbor, ME. $GILT^{-/-}$ mice that had been backcrossed to B6 mice for a minimum of 8 generations (16) were maintained in a colony at Yale University. B cell-deficient μMT mice were originally obtained from Jackson Laboratories and maintained in a colony at Yale University. All mice were treated according to protocols approved by the Yale University Animal Care and Use Committee.

Myelin oligodendrocyte glycoprotein

MOG protein was prepared as previously reported with bacteria expressing the extracellular domains of MOG from rat, human (C. Linington; University of Aberdeen, Aberdeen, Scotland), or mouse (M. Gardinier, University of Iowa, Iowa City) (17) (Fig. 1).

Rodent $MOG_{35–55}$ peptide was synthesized by the W.M. Keck Biotechnology Resource Center at Yale University. The sequence of the rodent peptide (rat and mouse MOG proteins are identical in this region) is MEVGWYRSPFSRVVHLYRNGK.

A series of six additional overlapping mouse MOG peptides (p1–21, p15–35, p50–70, p67–87, p85–105 and p104–117) were synthesized by the W.M. Keck Biotechnology Resource Center at Yale University.

The sequences of the mouse overlapping peptides are:

MOG 1–21: GQFRVIGPGYPIRALVGDEAE;

MOG 15–35: LVGDEAELPCRISPGKNATGM;

MOG 35–55: MEVGWYRSPFSRVVHLYRNGK;

MOG 50–70: LYRNGKDQDAEQAPEYRGRTE;

MOG 67–87: GRTELLKETISEGKVTLRIQN;

MOG 85–105: IQNVRFSDEGGYTCFFRDHSY;

MOG 104–117: SYQEEAAMELKVED

EAE induction by active immunization

For EAE induction, female WT and GILT$^{-/-}$ mice were immunized by s.c. injections with either 300 μg of $MOG_{35–55}$, or 100 μg of recombinant MOG proteins in CFA (Difco, Detroit, MI) with 300 μg of *Mycobacterium tuberculosis* on d 0 and 7. Mice received i.p. injections of 500 ng of pertussis toxin (PTX) (List Biological Laboratories, Campbell, CA) on d 0 and 2.

Passive transfer of EAE with serum

A previously described protocol for passive transfer with serum was used (10). GILT$^{-/-}$ or μMT mice were immunized with a single injection of $MOG_{35–55}$ or rhuMOG in CFA on d 0, and injected with PTX on d 0 and 2. These manipulations do not induce EAE in these particular mouse strains, but do prime T cells, which is a necessity for the success of this protocol (18). Mice received four i.v. injections of 150 μl of pooled serum beginning at d 0 and at 3-day intervals for a total of 600 μl. The donor anti-sera were obtained from GILT$^{-/-}$ or C57BL/6 mice 14 days after immunization with rratMOG or rhuMOG in CFA. Control serum was obtained from non-immunized GILT$^{-/-}$ mice.

Clinical Disease Scoring

Mice were monitored daily for clinical signs of EAE. Clinical scores were based on a scale of 0–5, with a score of 0 indicating no disease and a score of 5 indicating death. A score of 1 indicates a limp tail, 2 indicates paresis or partial paralysis of the hind limbs, 3 indicates total hind limb paralysis, and 4 indicates fore and hind limb paralysis. Data are presented as mean clinical scores for each group, with dead animals given a score of 5 on the day of death. Disease onset was calculated as the average day of appearance of clinical signs. Disease indices for each group were calculated as: ((sum of the mean clinical scores)/(mean day of disease onset)) × 100.

Histopathologic Examination

The cellular composition of the infiltrates in the CNS of WT and GILT$^{-/-}$ mice, on d 20 after EAE induction with rratMOG, was evaluated. Mice were deeply anesthetized and intra-cardiac perfused with cold PBS. Spinal cords were removed and fixed in zinc-buffered Formalin. Tissues were paraffin embedded, sectioned, and stained with H&E by the Dermatopathology Laboratory at Yale University. Four slides were prepared of axial sections from both WT and GILT$^{-/-}$ spinal cord. Each slide held sections of tissue representing the cellular composition at 4 separate positions, 25um apart, along the length of spinal cord. A 100X oil immersion objective and scaling software was used to outline 4 distinct 50um^2 regions per section. A total of 16 regions were evaluated per mouse spinal cord. The number of cells with the appearance of small mononuclear cells or plasma cells were counted and compared between WT and GILT$^{-/-}$ mice.

Statistical analysis

Differences in disease onset and maximum disease scores and the cellular composition of the CNS were analyzed by Student's *t* test, using a *p* value of ≤0.05 as the threshold of significance.

Immunofluorescence

For immunofluorescence, spinal cords were removed and immediately frozen in OCT compound (Sakura Finetek, Torrance, CA) using a dry ice/methylbutane bath. Sections of 7 μm were cut onto poly-L-lysine coated glass slides (Sigma-Aldrich, St. Louis, MO), fixed in 100% cold acetone and stored at −70°C. Sections were blocked with 5% mouse serum/5% BSA in PBS, pH 7.4. Allophycocyanin (APC)- conjugated rat anti-mouse CD138 (BD Pharmingen, San Diego, CA) was diluted 1:250 in blocking buffer and incubated on sections for 1.5 h. Slides were washed 3X in PBS and then incubated with PE-conjugated rat anti-mouse CD38 (BD Pharmingen) diluted 1:250 in blocking buffer and incubated on sections for 1.5 h. Slides were washed 3X in PBS and then sections were counterstained with DAPI (Sigma-Aldrich) and mounted with Fluorosave (Calbiochem, San Diego, CA). Tissue sections were analyzed by fluorescence microscopy using a Carl Zeiss Microimaging, Inc. Axioskop microscope.

Proliferation Assays

Spleens were removed from GILT$^{-/-}$ or C57BL/6 mice. The dispersed, irradiated (2000 rad) cells were used as a source of APC. The antigen-specific T cell line was established from the lymph node (LN) cells of C57BL/6 mice immunized with $MOG_{35–55}$. These cells have been maintained in long-term culture with bi-weekly administration of recombinant human rIL-2, $MOG_{35–55}$ and irradiated C57BL/6 spleen cells as APC. LN cells were isolated from the peri-aortic and inguinal LN of GILT$^{-/-}$ mice immunized 10 d previously with a single injection of 100 μg of MOG protein in CFA in a total volume of 200 μl distributed in the rear flanks and at the base of the tail. Assay cultures contained 2×10^5 T cells and 6×10^5 APC per well, in triplicate, in a volume of 200 μl in 96 well flat bottom plates, in the presence of rratMOG (40 μg/ml) or peptides (20 μg/ml), as indicated, in RPMI supplemented with penicillin/streptomycin, fungizone, 10% FCS and 0.05 μM 2-ME.

Cultures were pulsed with 1.0 μCi of [^{3}H] TdR at 48 h, and harvested 24 h later with a TOMTEC (Hamden, CT) harvester. Thymidine incorporation was measured in a Wallac (Gaithersburg, MD) Microbeta 1450 liquid scintillation counter.

MOG Antibody ELISA

Nunc Maxisorp 96 well micro-titer plates (Macalaster Bicknell Co., Inc., New Haven, CT) were coated overnight at 4°C with 100 μl/well of extracellular recombinant mouse MOG protein at a concentration of 10 μg/ml in 0.05M carbonate buffer (pH 9.6). Plates were washed 3X with PBS, followed by three washes with PBS containing 0.05% Tween 20, then blocked at room temperature for 2 h with PBS containing 3% BSA. After 3 washes with PBS/Tween 20, serum samples were serially diluted in PBS/3%BSA and added to the wells, in triplicate. Plates were incubated for 4 h at room temperature. Following 3 washes with PBS/Tween 20, HRP-labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA) was added as the secondary antibody and incubated overnight at 4°C. Following 5 washes with PBS, plates were developed using TMB substrate (Sigma-Aldrich) and absorbance read at 450 nm on a PowerWave XS plate reader (Bio-Tek Instruments, Winooski, VT). Titers were determined with dilutions that resulted in two standard deviations above the highest reading of the negative control.

IgG Preparation and Purification

IgG was purified from the sera of the following groups of mice: C57BL/6, GILT$^{+/+}$ or GILT$^{-/-}$ immunized with rratMOG or GILT$^{+/+}$ immunized with rhuMOG. IgG from sera pooled from at least 10 mice collected 14 or 21 d after immunization were purified by protein G-Sepharose (Sigma-Aldrich) chromatography; purity and titer were assayed by SDS PAGE and ELISA. Three independent IgG preparations were used for each experiment.

OL Culture

Enriched populations of mature rat OLs were prepared and maintained (19, 20). Purified OLs were grown in defined modified N2 medium (21, 22) for 6–7 d to obtain MOG-expressing OLs.

Myelin Purification

Myelin was purified from postnatal d 35 C57BL/6J mouse brains (23)

MOG Cross-linking

Rat OL cultures were incubated for 30 min at 37°C with the different purified IgGs (100 μg/ml). MOG-anti-MOG complexes were then cross-linked with goat anti-mouse IgG as previously described(24, 25).

Antibody Binding

To estimate antibody binding ability, live OLs were incubated with 100 μg/ml of the different purified IgGs followed by fluorescently labeled secondary antibodies. To examine changes in morphology, mature OLs were stained live with O4 antibody as described (24).

The diameter of randomly chosen cells (100 cells per experiment) was determined by using a calibrated microscopic grid.

Cell Lysate Preparation and Detergent Extraction

OLs were scraped into 150 mM NaCl, 5 mM EDTA, 25 mM Tris-Cl buffer (pH 7.5) containing 1 mM PMSF, 10 g/ml leupeptin-aprotinin, 50 mM NaF, 10 mM NaP2O7, 1 mM Na o-Vanadate, and 1% Triton X-100, centrifuged to separate them into detergent insoluble pellet and detergent-soluble supernatant fractions, and processed for SDS-PAGE (24).

SDS PAGE and Immunoblot Analysis

Samples of purified myelin (10 μg), or equal volumes of soluble and insoluble fractions of detergent extracts were solubilized in 50 mM Tris HCl, pH 6.8, 2.5% glycerol, 5% SDS, 4 M urea, 0.01% bromophenol blue, 10 mM DTT for SDS PAGE (24), immunoblotted with 2 μg/ml of purified IgGs followed by incubation with HRP-labeled secondary antibody.

RESULTS

Mice deficient in GILT are relatively resistant to $MOG_{35–55}$-induced EAE but are more susceptible than wild type mice to rat MOG protein-induced EAE

We hypothesized that $GILT^{-/-}$ mice with defective antigen processing would be unable to process MOG protein to $MOG_{35–55}$ and would thus be resistant to EAE after injection of rratMOG. We also hypothesized that $GILT^{-/-}$ mice immunized with $MOG_{35–55}$ peptide, which does not require processing, would be able to respond to the encephalitogenic peptide and might develop EAE. The opposite occurred. $GILT^{-/-}$ mice were nearly completely resistant to EAE after injection of $MOG_{35–55}$ but were slightly more susceptible than WT (C57BL/6 or $GILT^{+/+}$ littermates) mice to EAE induced by the extracellular domain of rratMOG (Fig. 2 and Table I). After immunization with $MOG_{35–55}$, fewer $GILT^{-/-}$ mice developed clinical disease, none died, and the mean maximum disease score was lower in $GILT^{-/-}$ mice (0.87) than in WT (3.25) (p <0.007), as was the disease index (69.4 vs. 445.6). Histological analysis of spinal cords at 40 d post immunization confirmed these observations. As expected from the clinical scores, there was more intense inflammation in the spinal cords of WT mice immunized with the peptide than those of $GILT^{-/-}$ mice (data not shown). These data indicate that $MOG_{35–55}$ is relatively poorly encephalitogenic in $GILT^{-/-}$ mice.

WT mice that had been injected with rratMOG had a disease index of 541.7 and a mean maximum disease score of 3.25, similar to our previous studies (9). On the other hand, identically injected $GILT^{-/-}$ mice had a disease index of 774 with a mean maximum disease score of 4.25 (p <0.022), indicating a somewhat more severe disease. At d 20, there was a striking difference in the histological appearance of the infiltrates (Fig. 3). Those from WT mice (Fig. 3*A*, 3*C*) had the previously described typical appearance of an intense mononuclear infiltrate with lymphocytes, macrophages, activated microglia, T cells and very few B cells (9), while the CNS of $GILT^{-/-}$ mice immunized with rratMOG had fewer (Fig. 3*B*, 3*D*) and larger infiltrating cells. Most of those cells had the large clock-faced appearance of plasma cells (Fig. 3*D*); cells rarely found in the CNS of $MOG_{35–55}$-

immunized WT mice. Reactivity with antibodies to the plasma cell markers, CD38 and CD138 (Fig. 3*F*) confirmed their identity. A measurement, of the mean number of infiltrating cells in a 50um^2 section of spinal cord, confirmed significantly fewer mononuclear cells (GILT$^{-/-}$ = 0.88 ±1.1 vs. WT = 7 ±3.32; $p < 0.001$) and significantly more plasma cells (GILT$^{-/-}$ = 3 ±1.1 vs. WT = 0.44 ±0.63; $p < 0.001$) in GILT$^{-/-}$ mice compared to WT mice (Fig. 4). These data suggested that the mechanism of EAE in rratMOG-immunized GILT$^{-/-}$ mice might differ from that of WT mice.

Exogenous MOG$_{35–55}$ is immunogenic in GILT$^{-/-}$ mice but GILT$^{-/-}$ APCs cannot generate MOG$_{35–55}$ peptide from rat MOG protein

We have previously shown that WT C57BL/6 mice immunized with MOG$_{35–55}$ develop EAE and their LNC proliferate to the peptide (9) in confirmation of data from other investigators (11). We also showed that LNC from rratMOG-immunized WT mice proliferate in response to that protein $_{-/-}$ and to mouse MOG (9). Even though MOG$_{35–55}$ was only weakly encephalitogenic in GILT mice, it was still possible that the peptide was immunogenic in these mice. To test this hypothesis, WT and GILT$^{-/-}$ mice were immunized with MOG$_{35–55}$ and after 10 d, their spleen cells were re-exposed in vitro to that peptide. As shown in Figure 5, GILT$^{-/-}$ spleen cells (Fig. 5*B*), like those from WT mice (Fig. 5*A*), which include T cells and APCs, proliferated under these conditions, suggesting that in GILT$^{-/-}$ mice, MOG$_{35–55}$ is immunogenic even though, in these mice, it is very weakly encephalitogenic. However, in contrast to WT mice, spleen cells from GILT$^{-/-}$ mice immunized with rratMOG did not proliferate when cultured with MOG$_{35–55}$ in vitro (Fig. 5*A*, 5*B*). This suggested that GILT$^{-/-}$ APC could not process rratMOG to MOG$_{35–55}$. Evaluating the ability of GILT$^{-/-}$ spleen cells to present rratMOG to a WT T cell line specific for MOG$_{35–55}$ tested this possibility. Irradiated GILT$^{-/-}$ spleen cells were incubated with T cells in the presence of rratMOG. Whereas APCs from WT mice induced a striking proliferative response to antigen (CPM= 3546 ±745; stimulation index (SI) =74), those from GILT$^{-/-}$ mice effected a response only slightly higher than background (CPM=128 ± 89; SI=3) (Fig. 6). These data indicate that GILT$^{-/-}$ APCs cannot process the exogenous MOG protein to a peptide recognized by T cells specific for MOG$_{35–55}$, while they are capable of presenting the processed peptide. These data provide a possible explanation for the inability of MOG$_{35–55}$ immunization to induce EAE in these mice because it is likely that they cannot process the endogenous MOG protein to MOG$_{35–55}$, a requirement for recognition by the immunized T cells.

A switch in the dominant epitope occurs in GILT$^{-/-}$ mice immunized with rat MOG protein

Next we tested whether T cells from GILT$^{-/-}$ mice recognized the same peptides as WT T cells after MOG immunization. Here we used mouse peptides because we were particularly interested in the response to the endogenous MOG. As indicated in Figure 1, rat and mouse peptide 35–55 are identical, with only a total of 6 residue differences between the ECD of mouse and rat MOG protein. LN cells isolated from WT mice 10 d after immunization with rratMOG proliferated in vitro when exposed to rratMOG and to MOG$_{35–55}$, but not to other mouse MOG peptides (Fig. 7*A*). On the other hand, LN cells isolated from rratMOG-immunized GILT$^{-/-}$ mice proliferated to rratMOG as expected, though not when exposed to MOG$_{35–55}$ (Fig. 7*B*). However, they did proliferate variably to several other mouse peptides,

including peptides 67–87, 85–105, and 104–117. The response was most marked to peptide 104–117. Thus, there is an epitope switch from the normally encephalitogenic peptide 35–55 to other peptides that are not usually recognized by WT T cells in the context of MOG protein immunization (9, 12). These data suggest that GILT$^{-/-}$ mice, even though they cannot process MOG to generate peptide 35–55, can generate and respond to other epitopes outside the internal disulfide bond.

Rat MOG-immunized GILT$^{-/-}$ mice generate pathogenic antibodies that transfer EAE

In our previous studies, we demonstrated that WT mice immunized with recombinant rat or human MOG (rhuMOG) generated antibodies that bound MOG. However, only antibodies from rhuMOG-immunized mice induced EAE upon transfer to rhuMOG-immunized B cell-deficient μMT mice that do not normally develop EAE. These experiments also indicate that rhuMOG immunization does not generate reactivity to the predominant mouse MOG$_{35–55}$ epitope, but that priming of T cells does occur, resulting in opening the blood-brain barrier to the pathogenic antibody. Even though WT mice immunized with MOG$_{35–55}$ or rratMOG generate anti-MOG antibody, their sera are non-pathogenic, in that they do not transfer EAE into primed μMT mice (10). This is because the antibodies in these sera only recognize recombinant MOG and not native glycosylated MOG. The interpretation of the previous studies was: if pathogenic CD4 T cells cannot recognize a T cell encephalitogenic epitope, a switch in pathogenic mechanism occurs, resulting in a B cell-dependent, antibody-mediated disease. Here, we asked whether such a switch in pathogenic mechanism, from a predominant inflammatory T cell mediated disease to an antibody-mediated disease, occurred in GILT$^{-/-}$ mice immunized with rratMOG. First, we measured the titer of antibodies to mouse MOG in sera from rratMOG-immunized WT and GILT$^{-/-}$ mice. Both WT and GILT$^{-/-}$ mice produced high titer anti-mouse MOG antibody after immunization with rratMOG (Fig. 8). Pooled sera from rratMOG-immunized WT and GILT$^{-/-}$ mice, each revealed a titer of greater than 1:25,600 when tested against mouse MOG protein. Non-immune serum was negative for anti-MOG antibody.

We employed the same logic as in our previous experiments (10). That is, we hypothesized that the mechanism of EAE in rratMOG-immunized GILT$^{-/-}$ mice was antibody-mediated and that sera from such mice should induce EAE in mice primed in such a way that they would develop only minimal EAE. Thus, we transferred serum from GILT$^{-/-}$ or WT mice immunized with rratMOG to MOG$_{35–55}$ primed GILT$^{-/-}$ mice (Fig. 9*A*). Sera from rratMOG-immunized GILT$^{-/-}$ mice transferred EAE to MOG$_{35–55}$ primed GILT$^{-/-}$ mice, but sera from rratMOG-immunized WT mice did not (Fig. 9*B*). We also transferred serum from rratMOG-immunized GILT$^{-/-}$ mice to rhuMOG primed B cell deficient μMT mice. As reported in our previous studies, sera from WT mice immunized with rratMOG did not transfer EAE to primed μMT mice deficient in B cells (data not shown). However, sera from GILT$^{-/-}$ mice immunized with rratMOG transferred EAE to 2/5 rhuMOG primed μMT mice (Supplemental Table I). These data, taken together, indicate that immunization with rratMOG induces a pathogenic antibody in GILT$^{-/-}$ mice.

Serum from rat MOG-immunized GILT$^{-/-}$ mice recognizes native MOG

We have published several papers concerning the ability of some antibodies to bind to oligodendrocytes. Anti-MAG antibodies bind to OLs; leading to repartitioning into a detergent insoluble fraction, signaling changes, but no modifications in cell morphology (26). On the other hand, we noted properties of pathogenic antibodies from WT rhuMOG-immunized mice that were not exhibited by sera from WT mice immunized with rratMOG. These included binding to native glycosylated MOG revealed by Western blots, binding to cell surface MOG, and after cross-linking, the induction of morphological changes in the OLs and repartitioning of MOG into lipid rafts as indicated by a switch from a soluble to an insoluble (pellet) form (10). Here we evaluated whether sera from GILT$^{-/-}$ mice immunized with rratMOG also possessed these properties. First, we evaluated IgG in Western blots to determine if sera from rratMOG-immunized mice bound to native mouse myelin. As indicated in Figure 10*A*, IgG from immunized WT (B6 or GILT$^{+/+}$ 8th backcross littermates) did not bind to native mouse myelin (Fig. 10*A* i, ii). However, IgG from WT mice immunized with rhuMOG did bind to this native MOG (Fig. 10*A* iv), as expected from our previous studies (10). Serum from rratMOG-immunized GILT$^{-/-}$ mice also bound native MOG (Fig. 10*A* iii), in contrast, to the results with sera from WT mice. We next tested whether these antibodies could bind to OLs. Only IgG reactive to native mouse MOG bound to the cell surface of OLs. These binding sera were from WT mice immunized with rhuMOG (Fig. 10*B* iv) or from GILT$^{-/-}$ mice immunized with rratMOG (Fig. 10*B* iii). As in our previous studies, when cross-linked with antibody to mouse IgG, these sera induced a switch in the location of MOG from the soluble to the pellet form (Fig. 11*A*) (10). Furthermore, changes in the morphology of OLs were apparent with sera from GILT$^{-/-}$ mice immunized with rratMOG (Fig. 11*B* iii). However, sera from rratMOG-immunized WT mice did not bind native MOG (Fig. 10*A* i, ii), bind OLs (Fig. 10*B* i, ii), induce cross-linking and repartitioning into lipid rafts (Fig. 11*A*), or effect morphological changes in OLs (Fig. 11*B* i, ii); all of which had been possible with sera from WT mice immunized with human MOG (10). These data, taken together, indicate that rratMOG immunization of GILT$^{-/-}$ mice, but not WT mice, generates antibodies that have properties similar to those from WT mice immunized with human MOG, including transfer of EAE, ability to bind to glycosylated MOG, ability to bind to the surface of OLs, and when cross-linked, induce changes in partitioning of MOG and shape changes in OLs.

DISCUSSION

We show here that a defect in a processing enzyme can result in either a diminished clinical disease after immunization with a peptide, or a switch in pathogenic mechanism to an antibody mediated disease after immunization with a protein. The data in this paper address two basic issues in autoimmunity: 1) what are the antigen processing requirements for an endogenous antigen when immunizing with an exogenous antigen? 2) what are the consequences of immunization with an exogenous antigen that cannot be processed to an encephalitogenic peptide? We have addressed these issues experimentally and shown that: 1) the endogenous antigen most likely also needs to be processed to yield an encephalitogenic peptide; 2) an individual is capable of recognizing new peptides within the protein and switching to an entirely different pathogenic mechanism. We believe that these

results have profound implications for understanding any autoimmune disease in the highly out-bred human population.

We have investigated the role of GILT in the context of exogenously administered and endogenously processed antigens and analyzed an autoimmune disease that requires recognition of an endogenous CNS antigen. The data indicating that immunization of GILT$^{-/-}$ mice with MOG$_{35–55}$ does not induce a robust EAE are consistent with the concept that processing of the endogenous MOG protein is defective in these mice that are unable to reduce thiol bonds. Even though the original experiments defining the role of GILT in processing and presentation of disulphide linked proteins were carried out with exogenously administered antigens that had no endogenous counterpart (16), later experiments did evaluate the role of GILT in presentation of endogenous antigens. Our data confirm the observation that the provision of GILT to melanoma cells allowed them to present endogenous antigens (27) indicating that GILT is not only involved with processing of exogenous proteins.

Our studies revealed that MOG$_{35–55}$ is immunogenic but not encephalitogenic in GILT$^{-/-}$ mice. It is likely that the initial presentation of the peptide occurs through binding to MHC class II on an APC in the LN, likely a DC or macrophage and would not require further processing. However, administration of the peptide in a normally encephalitogenic form with CFA and PTX to GILT$^{-/-}$ mice resulted in a much reduced clinical score compared to WT mice. This strongly suggests that the endogenous MOG protein must be processed to cause disease and that this depends on GILT. These observations support and extend previous studies that evaluated the role of the class II processing pathway in EAE. Slavin and colleagues evaluated the role of invariant chain (Ii) and H-2M, two molecules involved in class II biosynthesis and endocytic processing. They found that splenic APCs could present MOG$_{35–55}$ to peptide-specific T cells in vitro, but were incapable of presenting native MOG from mouse brain. Furthermore, mice deficient in either Ia or H-2M were resistant to EAE induced by MOG$_{35–55}$ or recombinant MOG. Ii$^{-/-}$ or DM$^{-/-}$ mice were resistant to adoptive transfer of EAE from WT mice (28). Tompkins and colleagues confirmed these findings and also showed that mice deficient in CIITA, which have profound defects in class II and processing proteins, or DM or Ii were also resistant to clinical signs after administration of MOG$_{35–55}$ (29). Furthermore, even though T cells are primed in these immunized, class II deficient mice, as was the case in GILT$^{-/-}$ mice, there was no sign of CNS infiltration, suggesting the necessity for processing of the endogenous MOG protein, likely in the CNS.

In contrast to the results obtained with MOG$_{35–55}$ immunization, GILT$^{-/-}$ mice immunized with rratMOG exhibited a robust EAE. However, there were three important differences in this disease from that seen after rratMOG or MOG$_{35–55}$ immunization of WT mice. These differences included the cellular composition of the CNS infiltrates, epitope recognition, and pathogenic mechanism. The cellular composition in the CNS of GILT$^{-/-}$, but not WT mice included many cells likely to be plasma cells. The function of these cells has not been completely evaluated, but it is possible that the CNS is an ectopic or tertiary lymphoid organ (30) in these mice, serving as a site of B cell activation in germinal centers. Another difference was the loss of recognition of the dominant MOG$_{35–55}$ and the generation of

recognition of other epitopes. One of these (104–117) is completely outside the region defined by the disulphide bond; another (85 to 105) encompasses the bond, and the third (67–87) is actually within the area. It is possible that fine structure mapping of the 85–105 epitope will reveal that the Class II binding sequences are actually outside the disulphide bond. Additional investigation is necessary to determine how the epitope defined by 67–87 is available in the absence of disruption of the disulphide bond. It is possible that the three amino acid differences between mouse and rat MOG in this region influence this event. Thus, the ability to respond, or not, to these "new" epitopes is neither negatively nor positively correlated with amino acid differences between mouse and rat peptides. It should be noted that the response to a cysteine containing epitope by GILT$^{-/-}$ T cells is not unprecedented. Two cysteine containing epitopes of HEL, were recognized by T cells from GILT$^{-/-}$ mice, indicating that some epitopes exist that do not require reduction and re-folding before proteolytic processing (16). Our data are consistent with the previous demonstration that the CD4^{+} T cell response to the immunodominant TRP-1 epitope is diminished when GILT$^{-/-}$ dendritic (DC) cells are used as APCs to present this endogenous antigen (27). Mass spectrometry analysis, of MHC class II associated self-peptides from GILT$^{-/-}$ and WT splenocytes, suggested that self-peptides are more abundantly expressed by GILT$^{-/-}$ splenocytes, and that the diversity of the MHC class II-bound self-peptides is moderately affected by the absence of GILT (31). We assume that the initial presentation of both MOG$_{35–55}$ and rratMOG normally occurs by GILT expressing macrophages and DCs in the draining LNs. In the case of rratMOG, GILT$^{-/-}$ APCs will process the exogenous antigen to generate the epitopes noted in the text of this communication. The next step is for the antigen primed T cells to migrate to the CNS where the endogenous MOG is presented by APCs. Several putative APCs exist at that location, including resident microglia, OLs, and astrocytes. It is not yet known which of the putative CNS APCs express GILT necessary for processing of endogenous MOG. The switch in pathogenic mechanism to an antibody-mediated disease in rratMOG-immunized GILT$^{-/-}$ mice that do not recognize MOG$_{35–55}$, was not completely unexpected. A similar phenomenon is observed in WT mice immunized with rhuMOG with its proline rather than serine at position 42 (10). In that case and the present situation, the endogenous MOG$_{35–55}$ peptide is not seen in the CNS. This results in elicitation of a "fall back" mechanism-namely, recognition of a new epitope and generation of a pathogenic antibody. In both cases, this antibody recognizes native MOG, binds to OLs, and, upon cross-linking, induces changes in shape and in the location of MOG into detergent insoluble complexes. Our previously published data indicate that these alterations are accompanied by extensive alterations in the phosphorylation state of key proteins (24, 25, 32). These changes are dependent on Fc receptors and can occur even in mixed primary cultures in the absence of cross-linking antibody (32). In those situations, where the usual T cell encephalitogenic epitope is not recognized, these MOG-recognizing antibodies are key to the pathogenesis of EAE.

In conclusion, we have demonstrated that a defect in GILT, a crucial processing enzyme, gives rise to an alternative pathogenic mechanism in EAE. It is likely that subtle changes in antigen processing capability can yield different pathogenic mechanisms in any autoimmune disease in which reactivity to endogenous antigen is key.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

B6	C57BL/6 mice
DC	dendritic cells
EAE	experimental autoimmune encephalomyelitis
ECD	extracellular domain
GILT	gamma interferon-inducible lysosomal thiol reductase
GILT$^{-/-}$	mice deficient in GILT
HEL	hen egg lysozyme
LN	lymph node
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
OL(s)	oligodendrocyte(s)
PTX	pertussis toxin
rhuMOG	recombinant human MOG protein
rratMOG	recombinant rat MOG protein
MOG$_{35–55}$	rodent MOG peptide 35–55
WT	wild-type

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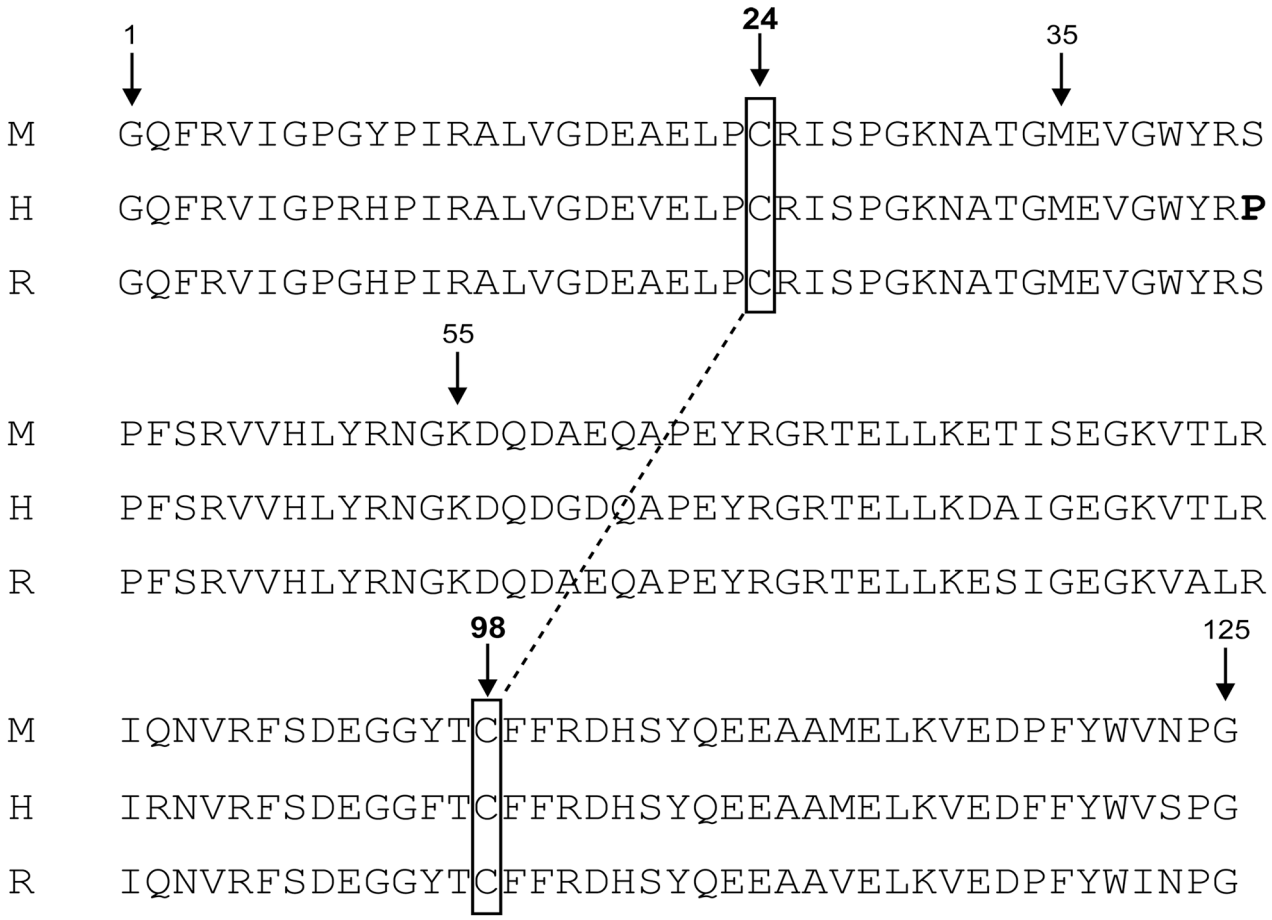

FIGURE 1.

A comparison of the sequences of the extracellular domain of mouse, human and rat MOG protein. Cysteine residues at positions 24 and 98 form an internal disulfide bond that includes the $MOG_{35–55}$ epitope. The sequence of the encephalitogenic peptide 35–55 is identical in the mouse and the rat protein and has been referred to as rodent MOG $peptide_{35–55}$. The sequence of the human peptide 35–55 differs from the rat and mouse peptide only at amino acid position 42, where a proline replaces the serine. This difference confers a significant change in mechanism of EAE from a T cell dependent disease to a B cell-antibody mediated disease.

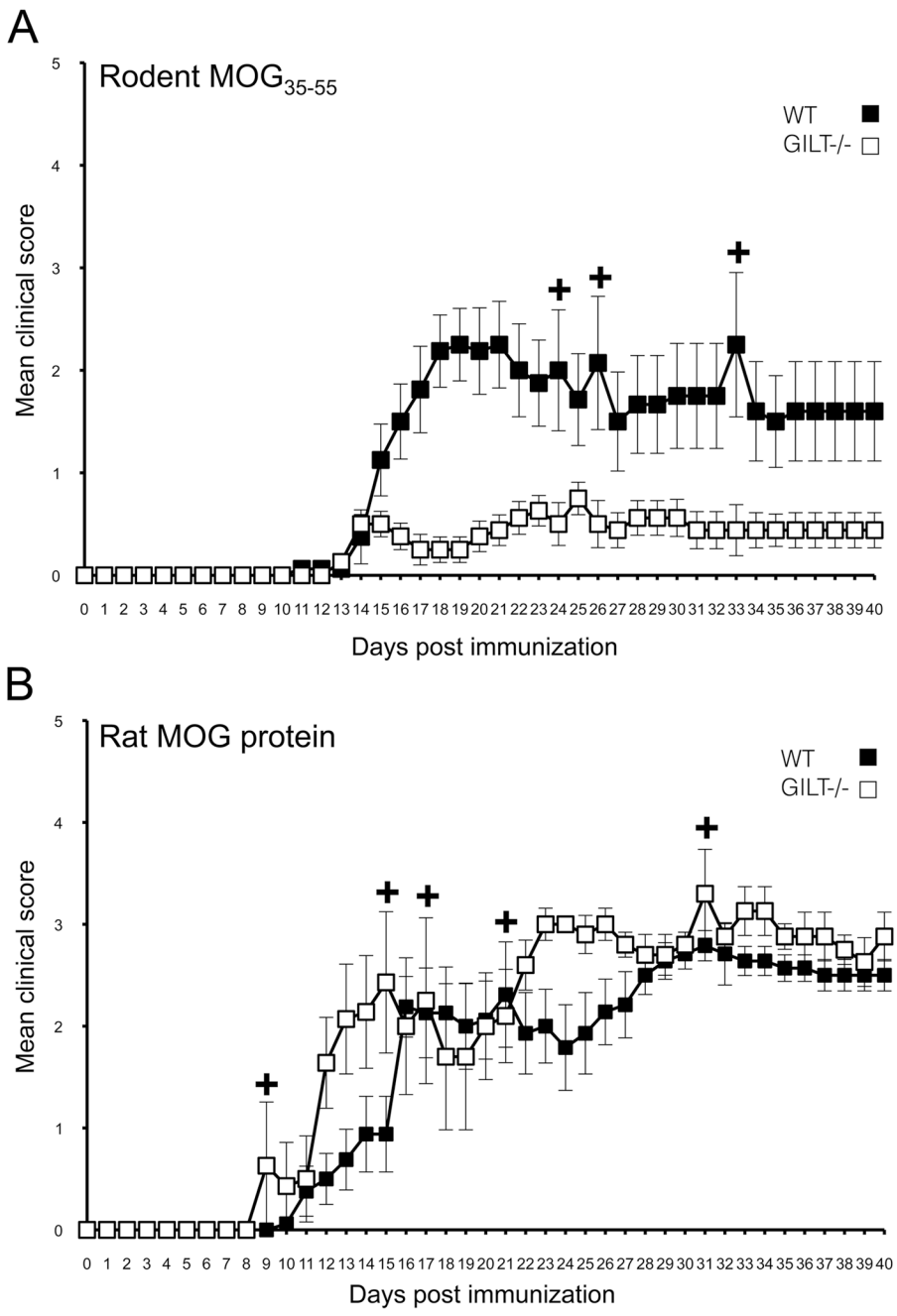

FIGURE 2.

Mice deficient in GILT are resistant to $MOG_{35–55}$-induced EAE but somewhat more susceptible than WT to rat MOG protein-induced disease. $GILT^{-/-}$ and WT mice were immunized with rodent $MOG_{35–55}$ (*A*) or rratMOG (*B*) in CFA to actively induce EAE as described in **Materials and Methods** and evaluated for clinical signs for 40 d. A cross (+) indicates the death of an animal in the group on that day. Error bars indicate ± SEM. Data represent the average of two (*B*) or three (*A*) independent experiments with a total of 8 pairs of mice.

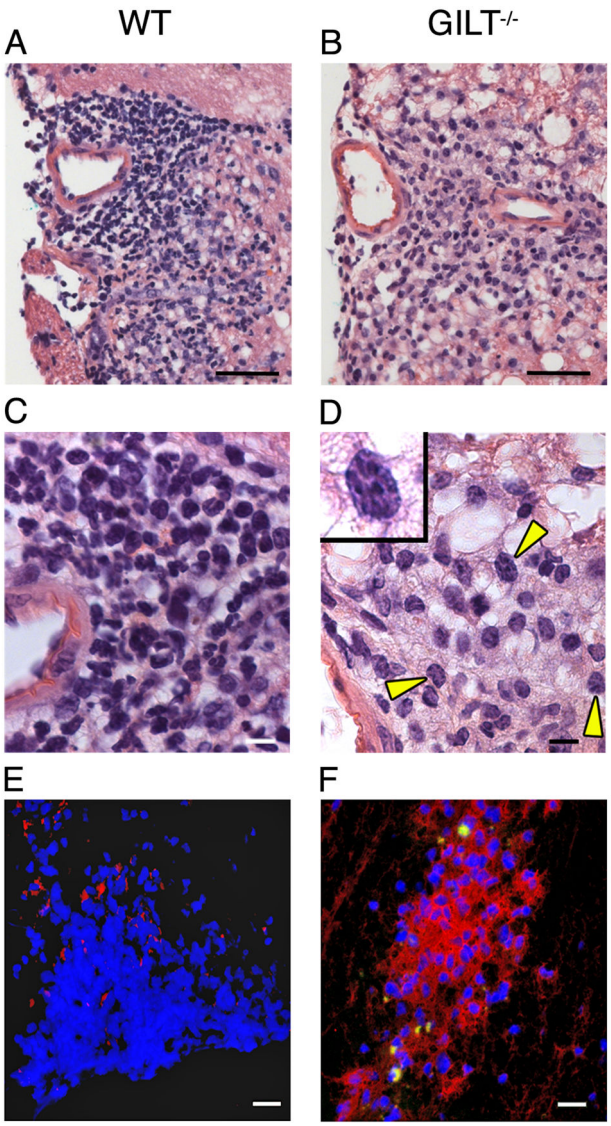

FIGURE 3.

Spinal cords from GILT$^{-/-}$ mice exhibit a different cellular composition with more plasma cells than those from WT mice. WT and GILT$^{-/-}$ mice were immunized with rratMOG in CFA and PTX. Spinal cords were removed on d 20 and analyzed by H&E for infiltrates (*A*–*D*). Arrows and insert indicate putative plasma cells (3*D*). Immunofluorescence staining (*E* and *F*) for CD38 (red) and CD138 (artificially designated green, and appearing yellow because of colocalization). Bars: (*A*, *B*) 50μm; (*C*, *D*) 25μm; (*E*, *F*) 20μm. Data are representative of three independent experiments.

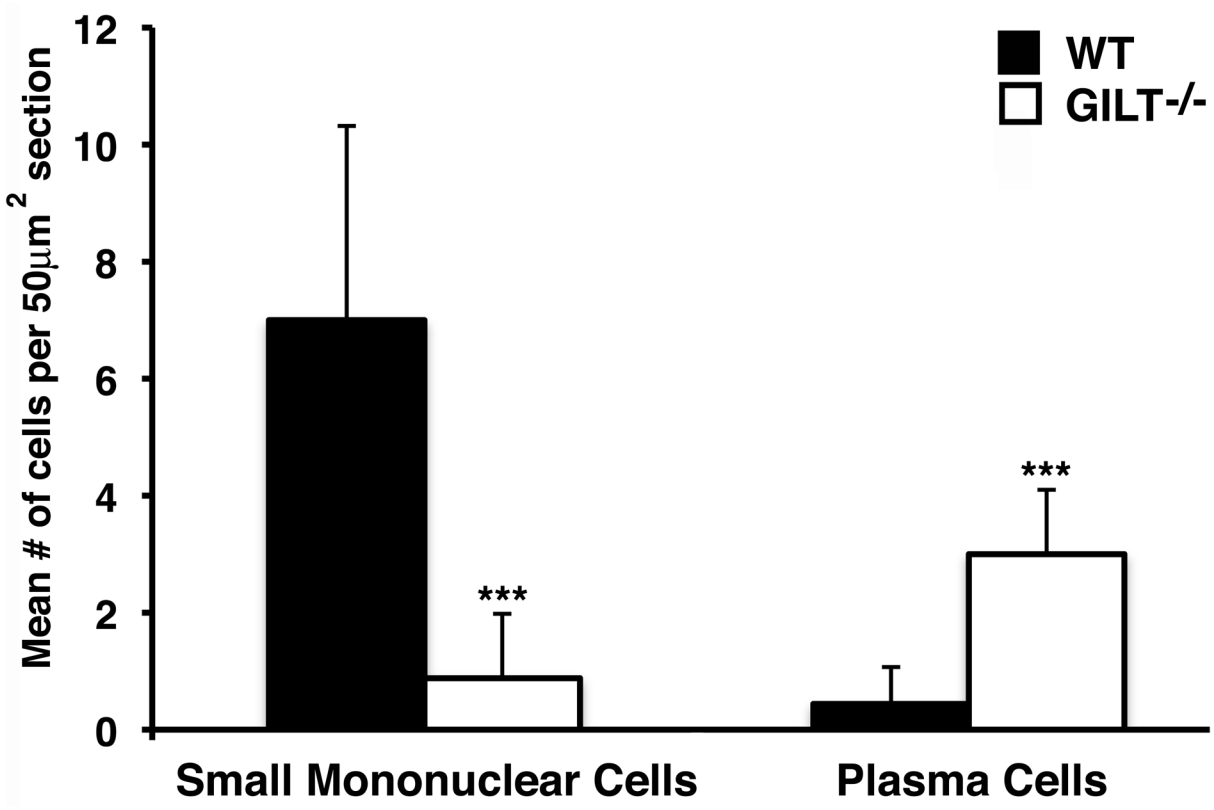

FIGURE 4.

Cellular infiltrate in the CNS of rratMOG-immunized GILT$^{-/-}$ mice shows a different composition than similarly immunized WT mice. Axial sections of spinal cord were stained with H&E. Small mononuclear cells and plasma cells in 50μm^2 regions of tissue were counted and compared. Data represent the mean ± SEM (***$p < .0001$ vs. WT). Data are representative of three independent experiments.

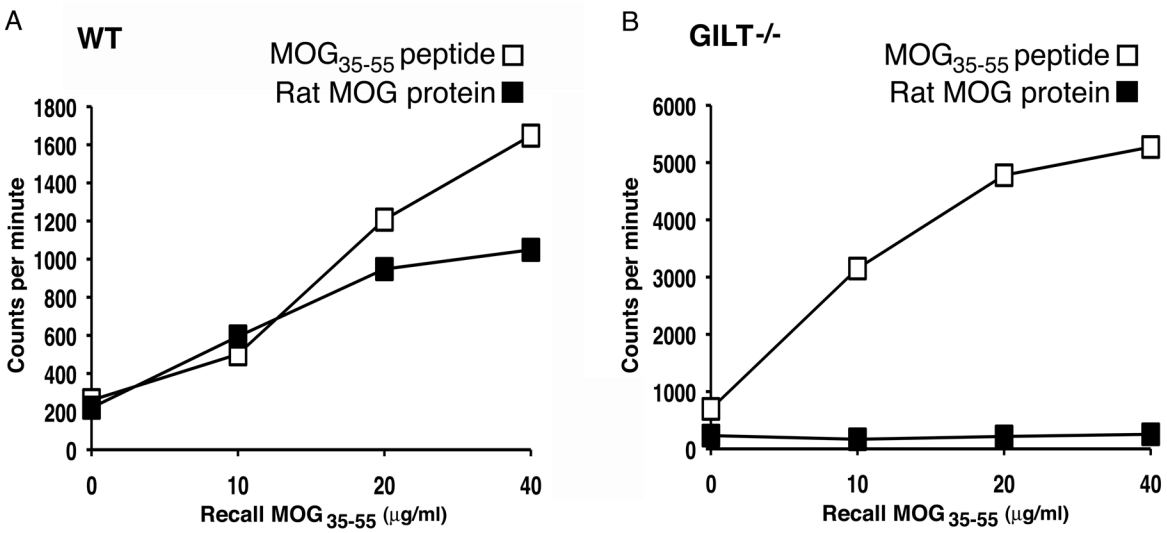

FIGURE 5.

Rat MOG protein does not prime GILT$^{-/-}$ mice for $MOG_{35–55}$ recall.

Spleen cells from WT (*A*) and GILT$^{-/-}$ (*B*) mice immunized 10 d previously with either $MOG_{35–55}$ peptide or rratMOG in CFA, were cultured with increasing concentrations of $MOG_{35–55}$ for 72 h. 1μCi of [^{3}H]-TdR was added to the culture for the final 24 h prior to harvest. Data are representative of two independent experiments.

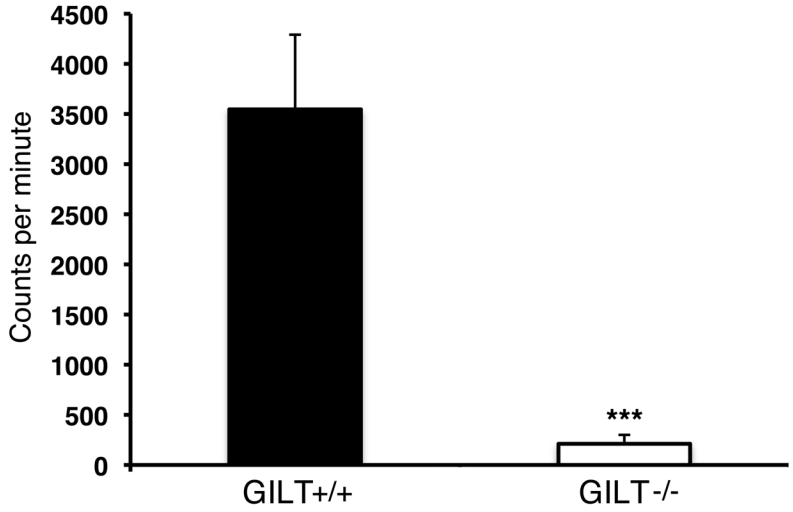

FIGURE 6.

GILT$^{-/-}$ APC cannot process rat MOG protein to $MOG_{35–55}$. Cells from an established rodent $MOG_{35–55}$-specific T cell line were cultured for 72 h with rratMOG and irradiated GILT$^{+/+}$ or GILT$^{-/-}$ spleen cell APC. 1μCi of [^{3}H]-TdR was added to the culture for the final 24 h prior to harvest. Data represent the mean CPM ± SEM (***p = .005 versus GILT$^{+/+}$ APC). Data are representative of two independent experiments.

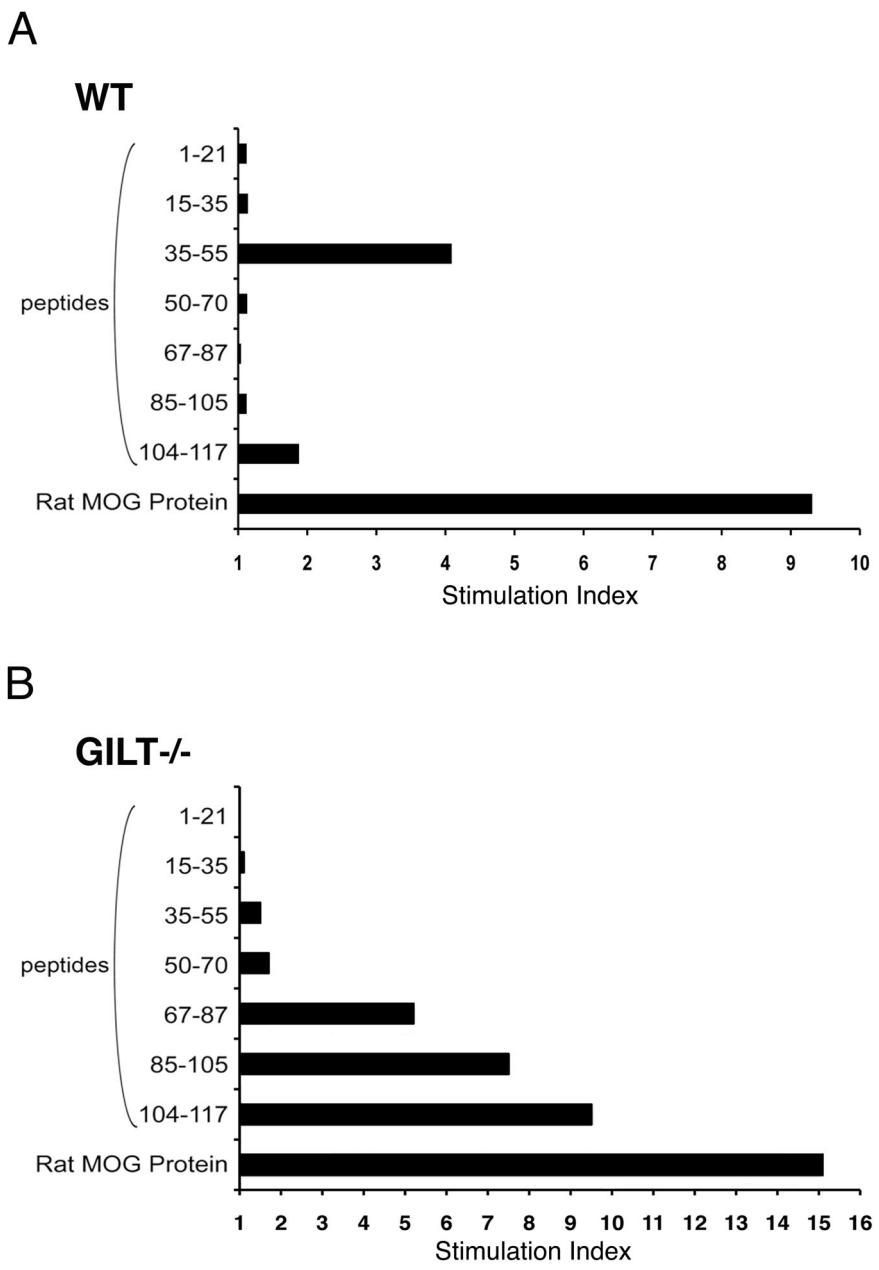

FIGURE 7.

A switch in predominant epitope occurs in rat MOG protein-immunized GILT$^{-/-}$ LN Cells. WT (*A*) and GILT$^{-/-}$ (*B*) mice were immunized with rratMOG as described in **Materials and Methods**. To determine epitope recognition, 10 d after immunization LN cells were cultured with rratMOG or a series of overlapping mouse MOG peptides. Data are presented as stimulation indices (stimulated/un-stimulated). For cultures with no added peptide was 45cpm for cells from WT mice and 125 cpm for cells from GILT$^{-/-}$ mice. Data are representative of two independent experiments.

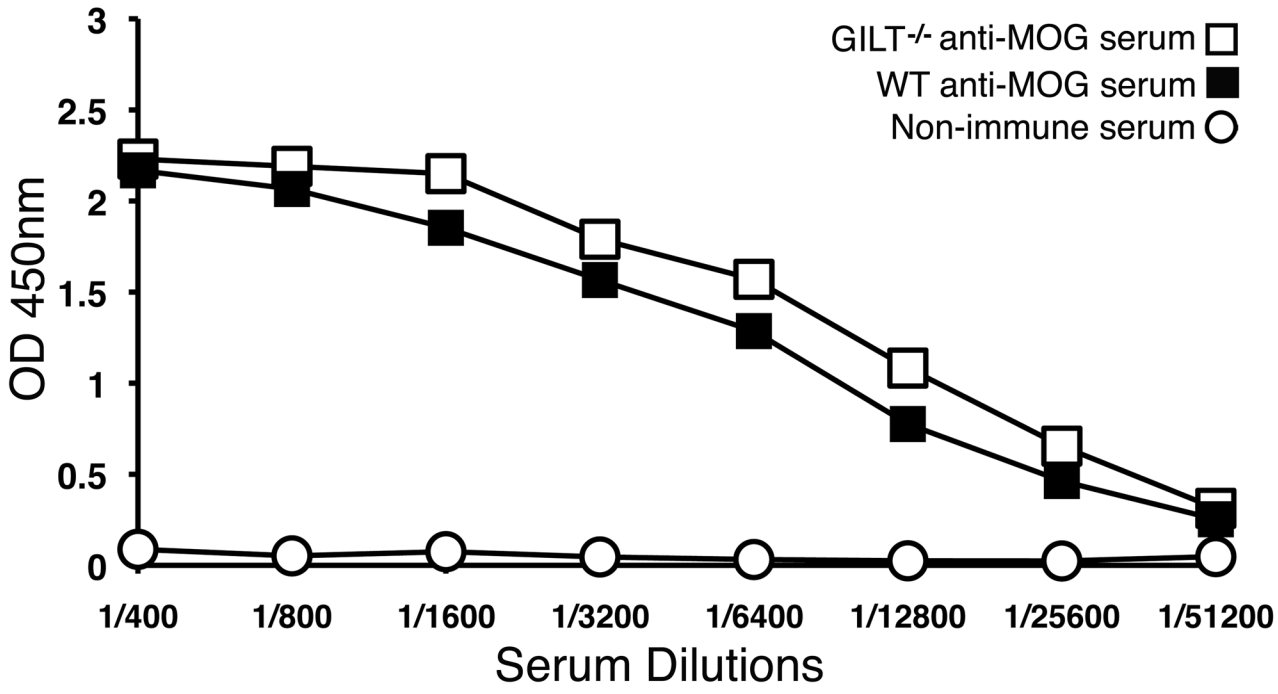

FIGURE 8.

GILT$^{-/-}$ and WT mice produce high titer anti-mouse MOG antibody after immunization with rat MOG protein. Pooled sera from WT or GILT$^{-/-}$ mice 14 d after immunization with rratMOG were analyzed by ELISA for total IgG and maintained a titer of antibody when diluted greater than 1:25,000. No MOG-specific antibodies were detected in samples from un-immunized mice.

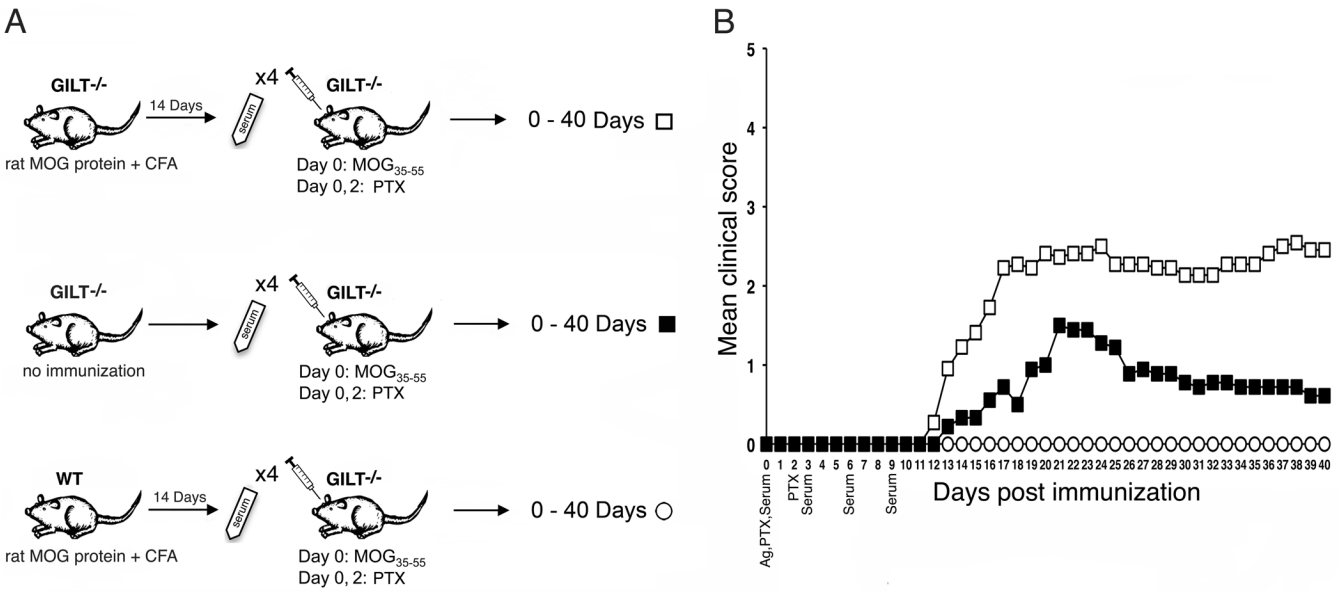

FIGURE 9.

Serum from rat MOG protein-immunized GILT$^{-/-}$ mice transfers EAE to GILT$^{-/-}$ mice.

A. Diagram of protocol for anti-rat MOG serum preparation in GILT$^{-/-}$ and WT mice and for peptide priming and serum transfer to GILT$^{-/-}$ mice.

B. Average daily clinical scores for GILT$^{-/-}$ mice that received 4 injections of 150μl anti-rat MOG serum (GILT$^{-/-}$ anti-serum (□), non-immune serum (■), or WT anti-serum (○)) at 3-day intervals (d 0,3,6,9), $MOG_{35–55}$ in CFA (d 0), and two injections of PTX (d 0, 2). Clinical signs were observed for 40 d. Mean maximum score (P= 0.001). These data are the average of two independent experiments.

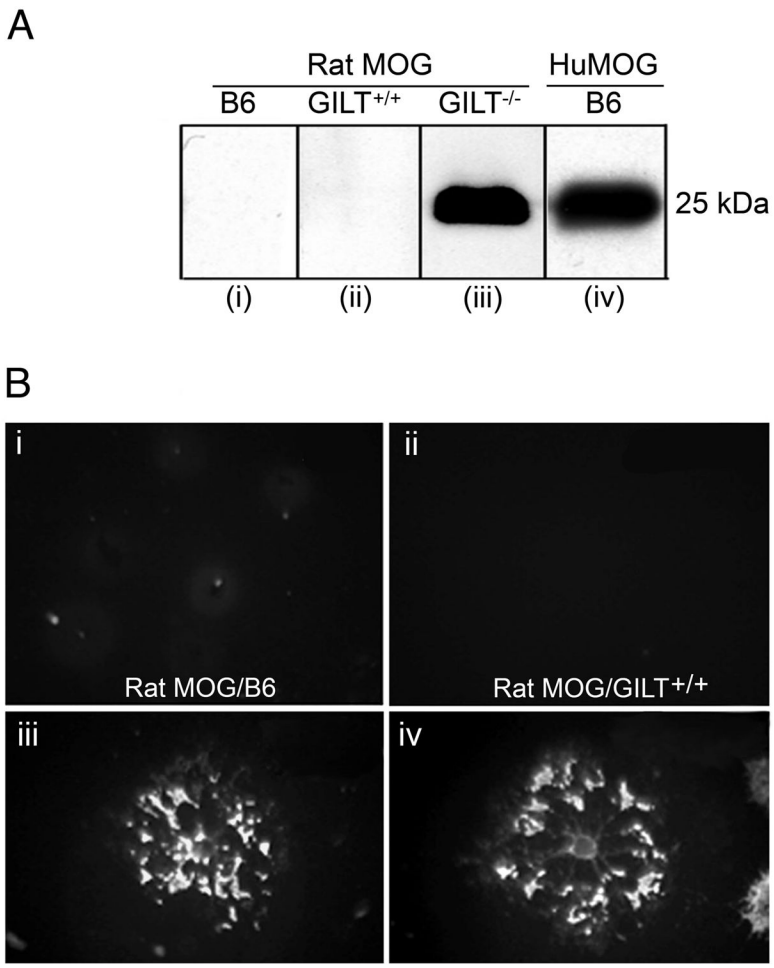

FIGURE 10.

IgGs from GILT$^{-/-}$, but not from WT (GILT$^{+/+}$ or C57BL/6) mice immunized with rat MOG protein bind native mouse myelin and oligodendrocytes. Mice were immunized with either rratMOG (*A* i, ii and iii) or rhuMOG (*A* iv). IgGs were purified from pooled sera collected from mice 14 d after immunization and (*A*) immuno-blotted on purified myelin or (*B*) incubated with OL cultures. (Bar, 5μm.) IgGs from rhuMOG-immunized WT mice bind to myelin and OLs (*A* iv; *B* iv), as do, those from rratMOG-immunized GILT$^{-/-}$ mice (*A* iii, *B* iii). IgGs from rratMOG-immunized WT mice do not (*A* i, ii; *B* i, ii). Three independent IgG preparations were used for each experiment.

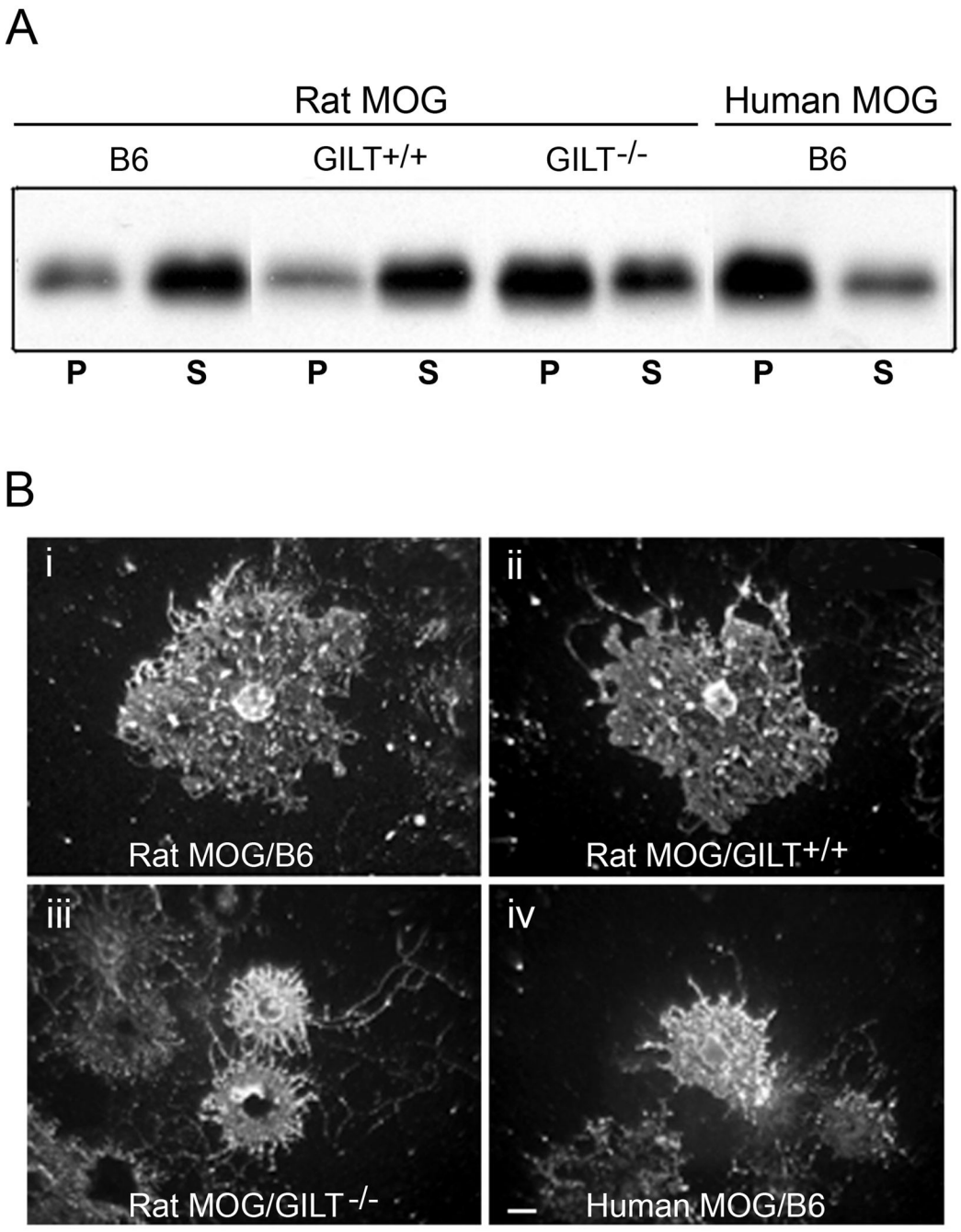

FIGURE 11.

MOG cross-linking of IgGs from GILT$^{-/-}$, but not WT (C57BL/6 or GILT$^{+/+}$) mice immunized with rat MOG protein induces MOG repartitioning into a detergent insoluble fraction and morphological alterations in oligodendrocytes.

(*A*) MOG immuno-blot of detergent insoluble pellet (**P**) or soluble (**S**) fractions from OL cultures incubated with IgGs from C57BL/6 or GILT$^{+/+}$ (B6, WT) or GILT$^{-/-}$ mice immunized with rratMOG or with IgGs from C57BL/6 mice immunized with rhuMOG protein. Treatment with IgGs from rratMOG-immunized GILT$^{-/-}$ mice or rhuMOG-

immunized WT mice induced repartitioning of MOG into the detergent insoluble pellet (**P**) (*B*) OLs were incubated with IgGs from C57BL/6 (*B* i), $GILT^{+/+}$ (*B* ii) or $GILT^{-/-}$ (*B* iii) mice immunized with rratMOG or C57BL/6 (*B* iv) mice immunized with rhuMOG; followed by, cross-linking with anti-mouse IgG and staining with mAb 04 to visualize OL morphology. (Bar, 5μm.) Only IgGs from $GILT^{-/-}$ mice immunized with rratMOG (*B* iii) and those from WT mice immunized with rhuMOG (*B* iv) induced retraction of OL processes. Three independent IgG preparations were used for each experiment. These experiments were performed as described in **Materials and Methods** and in previous publications (10, 24).

Table I

Evaluation of susceptibility of GILT$^{-/-}$ mice to MOG35–55 and Rat MOG protein

Immunogen	Incidence		Mortality		Mean day of onset (range)			Mean maximum clinical score (range)			Disease index day 40	
	GILT$^{-/-}$	WT	GILT$^{-/-}$	WT	GILT$^{-/-}$	WT	*P*	GILT$^{-/-}$	WT	*P*	GILT$^{-/-}$	WT
Rodent MOG 35–55	4/8	7/8	0/8	3/8	18 (13–24)	14 (11–16)	0.233	0.87 (0–3)	3.25 (0.5)	**<0.007**	69.4	445.6
Rat MOG Protein	8/8	8/8	4/8	1/8	13.2 (9–20)	12.6 (10–16	0.717	4.25 (3.5–5)	3.25 (2.5–5)	**<0.022**	774	541.7

There is a 50% reduction in incidence, 0% mortality, increased time before onset of symptoms, and a significant decrease in mean maximum disease score and clinical disease index in GILT$^{-/-}$ mice immunized with peptide compared to WT. In contrast, GILT$^{-/-}$ mice are equivalent to, or more susceptible than, WT mice to recombinant rat MOG protein-induced EAE.