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In Trans T Cell Tolerance Diminishes Autoantibody Responses and Exacerbates Experimental Allergic Encephalomyelitis

J. Jeremiah Bell,²* Rohit D. Divekar,* Jason S. Ellis,* Jason A. Cascio,* Cara L. Haymaker,* Renu Jain,* Danielle M. Tartar,* Christine M. Hoeman,* John C. Hardaway,* and Habib Zaghouani³*†

A number of Ag-specific approaches have been developed that ameliorate experimental allergic encephalomyelitis (EAE), an animal model for the human autoimmune disease multiple sclerosis. Translation to humans, however, remains a consideration, justifying the search for more insight into the mechanism underlying restoration of self-tolerance. Ig-proteolipid protein (PLP) 1 and Ig-myelin-oligodendrocyte-glial protein (MOG) are Ig chimeras carrying the encephalitogenic PLP 139–151 and MOG 35–55 amino acid sequence, respectively. Ig-PLP1 ameliorates EAE in SJL/J (H-2s) mice while Ig-MOG modulates the disease in C57BL/6 (H-2b) animals. In this study, we asked whether the chimeras would suppress EAE in F1 mice expressing both parental MHC alleles and representing a polymorphism with more relevance to human circumstances. The results show that Ig-MOG modulates both PLP1 and MOG peptide-induced EAE in the F1 mice, whereas Ig-PLP1 counters PLP1 EAE but exacerbates MOG-induced disease. This in trans aggravation of MOG EAE by Ig-PLP1 operates through induction of PLP1-specific T cells producing IL-5 that sustained inhibition of MOG-specific Abs leading to exacerbation of EAE. Thus, in trans T cell tolerance, which should be operative in polymorphic systems, can aggravate rather than ameliorate autoimmunity. This phenomenon possibly takes place through interference with protective humoral immunity. The Journal of Immunology, 2008, 180: 1508–1516.
for PLP1 peptide produced IL-5 cytokine which inhibited the production of MOG-specific Abs leading to exacerbation rather than amelioration of EAE. Thus, T cell tolerance that sustains amelioration of EAE in inbred mice evolved to negate protective humoral immunity in the more polymorphic F_1 mice and aggravates autoimmunity.

Materials and Methods

Animals

SJL/J (H-2^s), C57BL/6 (H-2^b), and B10.PL (H-2^b) mice were purchased from The Jackson Laboratory. F_1 (SJL/J × C57BL/6) and (SJL/J × B10.PL) mice were generated by breeding male SJL/J to female C57BL/6 and B10.PL, respectively. All mice were maintained in our animal care facility for the duration of the experiments. All experimental procedures were performed according to the guidelines of the institutional animal care committee.

Antigens

Peptides.

The peptides used in this study were purchased from Metabion and were HPLC purified to >90% purity. PLP1 peptide (HSLGKWLGH PDKF) encompasses amino acid residues 139–151 of PLP and is encephalitogenic in SJL/J mice (9). MOG peptide (MEVGWYRSPFSRVHLYRHKEQK) is encompassing amino acid residues 35–55 of MOG, is encephalitogenic in C57BL/6 and B10.PL mice (10). PLP peptide (NT WTTCSQSAFPSK) comprising amino acid residues 178–191 of PLP is also encephalitogenic in the SJL/J mouse (11). Myelin basic protein 3 (MBP3) peptide (VHFFKNIVTPRT) corresponds to amino acid residues 87–99 of MBP and is encephalitogenic in the SJL/J mouse (12). PLP-LR (HSLGKLLGRDPKF) is a mutant form of PLP-LR in which Trp144 and His147 were replaced with Leu and Arg, respectively, and serves as an antagonist to PLP-LR (13). Influenza virus hemagglutinin (HA) amino acid residues 110–120 peptide (SFERFEIFPKI) was used as a negative control (4).

CNS homogenate. Fifty frozen unstripped rat brains (Pelfreez Biologicals) were homogenized in PBS using a Waring blender and adjusted to 300 mg/ml with PBS (4, 5).

Ig chimeras.

Ig-PLP1 chimera, harboring PLP1 peptide within the H chain CDR3, has been shown to be effective against EAE when injected into mice in saline (14). Similarly, Ig-PLP harboring MOG 35–55 peptide (5), Ig-MBP3 carrying MBP87–99 (15), and Ig-MP2P2 encompassing PLP178–191 (16), have been shown to function as tolerogens when given to mice without adenoids (4, 5, 16). Ig-PLP-LR, which incorporates PLP1 antigen, serves as an antagonist for PLP-LR specific T cells (14). All Ig chimeras have, like Ig-PLP1, had the peptide of interest inserted within the H chain CDR3 region and were constructed using the general strategy for the IgG2b, k isoform of k-chain (9A13 as described previously (14)). In brief, the D segment was deleted from the CDR3 of the H chain V region and replaced with a nucleotide sequence that codes for the peptide using mutagenesis procedures described to those described for the generation of Ig-PLP1 (14). The resulting 9A13-3peptide chimeric IgG2b H chain was cotransfected with the parental 9A13 heavy chain into the non-Ig-producing SP2/0 myeloma B cell line, and the transfectoma cells producing complete Ig-PLP1 were selected with drugs as described previously (14).

Transfection, cloning, sequencing, and purification procedures for Ig-MOG, Ig-MBP3, Ig-MP2P2, and Ig-PLP-LR are similar to those used for Ig-PLP1 (5, 8, 14).

Aggregation of the Ig chimeras

The chimeras were aggregated by precipitation with 50%–saturated (NH_4)_2SO_4 as has been previously described (4). Because all chimeras are derived from the same Ig backbone and thereby comprise identical IgG2b isotypes, their Fc-associated functions will be similar.

Induction and scoring of EAE

Induction of EAE has been described previously (4, 5). Briefly, mice (6–8 wk old) were induced for EAE by s.c. injection in the footpads and at the base of the limbs with a 200–μl IFA/PBS (v/v) solution containing 6 mg of CNS homogenate, 100 μg of PLP1, or 300 μg of MOG, along with 200 μg of Mycobacterium tuberculosis H37Ra (Difco Laboratories). Six hours later, the mice were given i.v. either 200 (SJL/J × B10.PL) or 500 (SJL/J × C57BL/6) ng of purified Bordetella pertussis toxin (List Biological Laboratories). A second injection of B. pertussis toxin was given after 48 h. The mice were then scored daily for clinical signs of EAE as follows: 0, no clinical score; 1, loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or death.

Treatment of EAE

Standard treatment regimen. Mice were treated three times, 4 days apart with 300 μg of agg Ig chimeras at the first observation of clinical signs as described previously (4, 5). Typically, the treatments were given on days 13, 17, 21 postdisease induction by i.p. injection unless indicated otherwise.

Extended treatment regimen. In some experiments the F_1 (SJL/J × C57BL/6) mice received a prolonged treatment regimen consisting of 300 μg of agg Ig chimeras at the first observation of clinical signs and then every 4 days for the duration of the observation period.

In vivo antagonism of PLP1-specific T cells

In some experiments, antagonism of PLP1-specific T cells was conducted during treatment with agg Ig-PLP1. In this case, the mice were induced for EAE with MOG peptide and treated three times, 4 days apart with a mixture of 300 μg of agg Ig-PLP1 and 300 μg of agg Ig-PLP-LR at the first observation of clinical signs as described above.

In other experiments, antagonism of PLP1-specific T cells was conducted before induction of EAE or treatment with agg Ig-PLP1. Accordingly, the mice were given soluble (nonaggregated) Ig-PLP-LR on days −10, −6, and −3 before induction of EAE with MOG peptide. Soluble Ig-PLP-LR was used instead of agg Ig-PLP-LR because this form has been shown to antagonize PLP1-specific T cells (8, 14) and does not induce the production of IL-10 by APCs which could downregulate unrelated T cells by bystander suppression. When the signs of EAE were apparent, the mice were treated with agg Ig-PLP1 according to the standard regimen.

Detection of IFN-γ and IL-5 by ELISA

ELISA was performed according to BD Pharmingen standard protocol. The capture Abs were as follows: rat anti-mouse IFN-γ, R4-6A2 and rat anti-mouse IL-5, TRFK-5. The biotinylated anti-cytokine Abs were rat anti-mouse IFN-γ, XMG1.2, and rat anti-mouse IL-5, TRFK-4. The OD_540 was read on a SpectraMax 190 counter (Molecular Devices) and analyzed using SOFTmax PRO 3.1.1 software. Graded amounts of recombiant mouse IFN-γ and IL-5 (BD Pharmingen) were included for construction of a standard curve. The cytokine concentration in culture supernatants was extrapolated from the linear portion of the standard curve.

Inhibition of IFN-γ production by anti-CD4 Ab

F_1 (SJL/J × C57BL/6) mice were induced for EAE with MOG peptide and treated with agg Ig-PLP1 according to the standard treatment regimen. The spleen (SP) and lymph node (LN) anti-MOG IFN-γ responses were then analyzed 3 days after the final treatment. Accordingly, SP or LN cells (1 × 10^6 cells/well/100 μl) were stimulated with 30 μg/ml MOG peptide (50 μM/μl) and 20 μg/ml (50 μM/μl) of anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), or anti-H-2D^b (clone 28-14-8) Ab. For the combination of anti-CD8 plus anti-H-2D^b, 20 μg/ml of each was added. Rat IgG and mouse IgG were included as controls for Abs in matching doses. Cell cultures were incubated for 24 h at 37°C, after which culture supernatants were transferred to anti-IFN-γ-coated plates and cytokine was detected by ELISA as indicated above.

Detection of MOG-specific Abs by ELISA

For detection of anti-MOG Abs in the serum of MOG/EAE mice treated with agg Ig-PLP1, ELISA was used according to the following protocol. Fifty microliters of 0.1 M bicarbonate buffer containing 7 μg/ml MOG peptide was coated into 96-well plates and incubated overnight at 4°C. The plates were then washed and saturated with PBS-3% BSA. Subsequently, serial dilutions of serum were added and incubated overnight at 4°C. Total anti-MOG Abs were detected using anti-κ and anti-mouse Ig coupled to HRP from the Southern Biotechnology Associates cloning kit according to the manufacturer’s instructions. Class and subclass isotyping were performed in a similar manner using anti-IgG1, IgG2a, and IgG3, IgM, obtained from the Southern Biotechnology Associates cloning kit.

Statistical analysis

Statistical significance comparing EAE disease curves was analyzed by two-way mixed model ANOVA test using GraphPad Prism software.
disease severity. Control mice were given agg Ig-W, the parental MOG in saline at 4-day intervals and assessed for reduction in severity of EAE induced for EAE by s.c. injections of 6 mg of CNS homogenate along with 200 μg of H37Ra in a 200-μl suspension of PBS/IFA (v/v). When the clinical signs of EAE became evident (loss of tail tone), the mice were then treated i.p. with 300 μg of agg Ig-PLP1 (B and E), agg Ig-MOG (C and F), or control agg Ig-W (A and D) three times at 4-day intervals. The animals were then scored daily for the indicated periods of time.

Results

Ig-myelin chimeras reverse EAE in (SJL/J × C57BL/6)F1 mice

Reports indicated that agg Ig-PLP1 (14) and Ig-MOG (5) can reverse CNS homogenate-induced EAE involving diverse T cell specificities (4, 5). The mechanism underlying such effectiveness likely involves cytokine-mediated bystander suppression along with minimal costimulation (4, 5, 8). Because the previous studies were conducted in inbred animals with restricted MHC haplotypes, we sought to test the Ig-myelin system in a more polymorphic animal model, which would be more relevant to human circumstances. As such, we generated two types of F1 mice, an (SJL/J × B10.PL) and an (SJL/J × C57BL/6)F1 strain, and tested the agg Ig-myelin chimeras for suppression of EAE. Accordingly, the F1 female mice were induced for EAE with CNS homogenate, and when the clinical signs of disease became apparent, the animals were given three injections of 300 μg of agg Ig-PLP1 or agg Ig-MOG in saline at 4-day intervals and assessed for reduction in disease severity. Control mice were given agg Ig-W, the parental Ig backbone without any peptide insert. Fig. 1 shows that in the (SJL/J × B10.PL)F1 mice, those treated with agg Ig-W, similar to untreated animals, had a severe chronic form of EAE, while both agg Ig-PLP1- and agg Ig-MOG-treated animals had mild clinical signs of EAE (Fig. 1, upper panels). In contrast, in the (SJL/J × C57BL/6)F1 mice, while the CNS homogenate injection induced a milder monophasic form of EAE, the treatment with either agg Ig-PLP1, or agg Ig-MOG, had no significant effect in modulating disease (Fig. 1, lower panels). These results indicate that the ability of agg Ig-myelin chimeras to treat CNS homogenate-induced EAE depends on the genetic make up of the F1 mice and, probably, the resulting dominant epitopes.

Figure 1. Treatment of CNS homogenate-induced EAE in genetically distinct F1 mice with Ig chimeras shows differential efficacy. Groups of 6- to 8-wk-old (SJL × B10.PL) (A–C) or (SJL × C57BL/6) (D–F) F1 mice were induced for EAE by s.c. injections of 6 mg of CNS homogenate along with 200 μg of H37Ra in a 200-μl suspension of PBS/IFA (v/v). When the clinical signs of EAE became evident (loss of tail tone), the mice were then treated i.p. with 300 μg of agg Ig-PLP1 (B and E), agg Ig-MOG (C and F), or control agg Ig-W (A and D) three times at 4-day intervals. The animals were then scored daily for the indicated periods of time.

Figure 2. Agg Ig-PLP1 treatment exacerbates MOG peptide-induced EAE in SJL × C57BL/6 F1 mice. Groups (six to eight mice per group) of 6- to 8-wk-old (SJL × C57BL/6)F1 mice were induced for EAE by s.c. injections of 200 μl of PBS/IFA (v/v) containing 200 μg of H37Ra and either (A and B) 100 μg of PLP1 peptide or (C and D) 300 μg of MOG peptide. The mice were treated three times every 4 days with 300 μg of either agg Ig-PLP1 (A and C) or agg Ig-MOG (B and D) beginning at the first observation of clinical symptoms (day 13) and scored daily for the indicated periods of time. Groups of mice were left untreated (NIL) for comparison purposes.* Values of p < 0.05 as determined by two-way ANOVA described in Materials and Methods.
The indicated numbers represent the mean peptide and treated with agg Ig-PLP1 or agg Ig-MOG according to the standard legend to Fig. 2. These animals were induced for EAE with either PLP1 or MOG consecutive days. PBS/IFA (v/v) s.c. injections containing 300 daily for the indicated period of time. was left untreated (NIL) to serve as control. The mice were then scored ration of the experiment (extended treatment regimen) and a third group was given agg Ig-PLP1 every 4 days for the du-

was given agg Ig-PLP1 three times at 4 days interval (standard treatment regimen). Groups (six to eight mice per group) of 6- to 8-wk-old (SJL × C57BL/6)F1 mice were given 200 μg of MOG peptide and 200 μg of H37Ra. A group of mice was treated three times every 4 days with agg Ig-PLP1 (agg Ig-PLP1) beginning on the day when clinical signs of disease were first observed. An untreated group (NIL) was included for control purposes. The mice were then sacrificed at day 32 or 39 postdisease induction and their LN IFN-γ responses to MOG peptide were analyzed by ELISA. Each filled bar (■) represents the mean ± SD IFN-γ production from six to eight mice. Each open bar (□) represents the mean clinical score of the corresponding group of mice.

and recovered by day 32. To the contrary, mice which were treated with agg Ig-PLP1 showed exacerbated MOG/EAE with a mean disease score of 2.6 ± 0.5 and the mice did not recover for the entire 60-day monitoring period. These results indicate that agg Ig-MOG is effective at treating both PLP1- and MOG-induced EAE, but that agg Ig-PLP1, while effective at suppressing PLP1/EAE, exacerbates MOG-induced EAE and significantly delays spontaneous recovery.

To ensure that exacerbation of disease is not due to spreading to PLP1 epitope after completion of the treatment, the regimen was extended for a longer period of time and the animals were monitored for reversal of disease. As can be seen in Fig. 3, when compared with a group that was given only the standard three-injection regimen of agg Ig-PLP1, the mice that received the extended treatment regimen showed no reduction in disease severity. In fact, the mean maximal disease severity score was 2.4 ± 0.5 which is similar to the 2.5 ± 0.6 score of mice recipient of the standard regimen. Furthermore, both treatment regimens exacerbated the disease and resolution was delayed significantly. Indeed, whereas the untreated (NIL) group showed recovery by day 30 postdisease in-

![FIGURE 3. Exacerbation of MOG-induced EAE persists even with a continuous agg Ig-PLP1 treatment regimen. Groups (six to eight mice per group) of 6- to 8-wk-old (SJL × C57BL/6)F1 mice were given 200 μg of PBS/IFA (v/v) s.c. injections containing 300 μg of MOG peptide and 200 μg of H37Ra. At the onset of disease (loss of tail tone), a group of mice was given agg Ig-PLP1 three times at 4 days interval (standard treatment regimen), another group was given agg Ig-PLP1 every 4 days for the duration of the experiment (extended treatment regimen) and a third group was left untreated (NIL) to serve as control. The mice were then scored daily for the indicated period of time.](http://www.jimmunol.org/)

![FIGURE 4. agg Ig-PLP1 treatment prolongs MOG-specific IFN-γ production. Groups of 6- to 8-wk-old (SJL × C57BL/6)F1 mice were induced for EAE by s.c. injection of 200 μl of PBS/IFA (v/v) containing 300 μg of MOG peptide and 200 μg of H37Ra. A group of mice was treated three times every 4 days with agg Ig-PLP1 (agg Ig-PLP1) beginning on the day when clinical signs of disease were first observed. An untreated group (NIL) was included for control purposes. The mice were then sacrificed at day 32 or 39 postdisease induction and their LN IFN-γ responses to MOG peptide were analyzed by ELISA. Each filled bar (■) represents the mean ± SD IFN-γ production from six to eight mice. Each open bar (□) represents the mean clinical score of the corresponding group of mice.](http://www.jimmunol.org/)
The presence of anti-CD4, -CD8, or -H-2Db Abs were measured by experiments. Triplicate wells. The results are representative of two independent peptide and 20 μg/ml anti-CD8 Ab or anti-class I (H-2Db) Ab were added, no change responsible, at least in part, for mediating MOG responses. When anti-CD4 Ab was added to the cultures, the responses in both the SP (upper panel) and LN (lower panel) were significantly reduced, indicating that CD4 T cells are responsible, at least in part, for mediating MOG responses. When anti-CD8 Ab or anti-class I (H-2Db) Ab were added, no change was seen when compared with that of the normal response (medium). Taken together, these results indicate that treatment with agg Ig-PLP1 sustains activation of MOG-specific CD4, not CD8, T cells.

Spreading to epitopes other than PLP1 and MOG is not responsible for exacerbation of MOG-induced EAE by treatment with agg Ig-PLP1

Exacerbation of EAE by agg Ig-PLP1 may have resulted from activation of unrelated T cells as a consequence of a pattern of epitope spreading (20–22) dictated by the F1 MHC polymorphism. To address this issue, we sought to induce EAE with MOG peptide as in Fig. 3. At the onset of clinical signs (day 13), the mice were given injections of (A) agg Ig-PLP1 + agg Ig-MOG, (B) agg Ig PLP1 + agg Ig-PLP2, or (C) agg Ig-PLP1 + agg Ig-MBP1. Each mixture contained 300 μg of agg Ig-PLP1 and 300 μg of the additional chimera. A group of mice treated with 300 μg of agg Ig-PLP1 alone was included for control purposes.

FIGURE 5. MOG-specific CD4, not CD8, T cells mediate the anti-MOG response upon agg Ig-PLP1 treatment of MOG-induced EAE. Groups of 6- to 8-wk-old (SJL × C57BL/6)F1 mice were induced for EAE with MOG peptide as in Fig. 3. The mice were treated three times every 4 days beginning at the onset of clinical signs with 300 μg of agg Ig-PLP1. An untreated control group was included for comparison. Three days after the final treatment, the mice were sacrificed and their (A) SP and (B) draining LN cells were stimulated with MOG peptide in the presence or absence of blocking anti-CD4, -CD8, or -H-2Db Abs. The SP and LN cells were used at 1 × 10^6 cells/100 μl/well. The stimulation used 30 μg/ml MOG peptide and 20 μg/ml anti-CD4, -CD8, or -H-2Db (anti-class I) Ab. Combination of Abs uses 20 μg/ml each. Rat and mouse IgG were included as controls for Abs in matching doses. Each bar represents the mean ± SD of triplicate wells. The results are representative of two independent experiments.

FIGURE 6. agg Ig-PLP1 treatment continues to exacerbate MOG-induced EAE when administered with Igs carrying other myelin epitopes. Groups of 6- to 8-wk-old (SJL × C57BL/6)F1 mice were induced for EAE with 300 μg of MOG peptide as in Fig. 3. At the onset of clinical signs, the mice were given injections of (A) agg Ig-PLP1 + agg Ig-MOG, (B) agg Ig PLP1 + agg Ig-PLP2, or (C) agg Ig-PLP1 + agg Ig-MBP1. Each mixture contained 300 μg of agg Ig-PLP1 and 300 μg of the additional chimera. A group of mice treated with 300 μg of agg Ig-PLP1 alone was included for control purposes.

Three treatment regimens were tested which include agg Ig-PLP1 in combination with agg Ig-MOG; agg Ig-PLP1 along with agg Ig-PLP2 (16), a chimera carrying the I-Aδ-restricted PLP2 peptide corresponding to amino acid residues 179–191 of PLP; and agg Ig-PLP1 combined with agg Ig-MBP3 (15) a chimera carrying the promiscuous amino acid sequence 87–99 of MBP. These regimens were applied in the form of a mixture of equal amounts of chimera (300 μg each) according to the standard three injections at 4-day intervals. A group of mice recipient of agg Ig-PLP1 alone was included to serve as a control. As can be seen in Fig. 6, none of the combinations were able to induce reduction in the severity or duration of the disease. Indeed, the mean maximal disease score was 3.3 ± 1.0 for the treatment with agg Ig-PLP1 plus agg Ig-MOG; 3.3 ± 1.0 for agg Ig-PLP1 plus agg Ig-PLP2; and 2.5 ± 1.0 for the treatment with agg Ig-PLP1 plus agg Ig-MBP3. This is similar to the 2.8 ± 1.0 score observed during treatment with agg Ig-PLP1.
alone. Moreover, the patterns of clinical signs were similar throughout the duration of the monitoring time period. Thus, the exacerbation of MOG/EAE by agg Ig-PLP1 is not due to spreading to other epitopes such as PLP2 or MBP3. Interestingly, even agg Ig-MOG when combined with agg Ig-PLP1 is no longer able to modulate the disease.

**Antagonism of PLP1-specific cells by Ig-PLP-LR modulates agg Ig-PLP1-mediated disease exacerbation**

Epitope spreading does not seem to be responsible for exacerbation of MOG-induced EAE by agg Ig-PLP1. Treatment with Ig-MOG combined with Ig-PLP1 does not modulate the disease possibly indicating that MOG-specific T cells may not be responsible for exacerbation of disease. Thus, it may be that agg Ig-PLP1 in the context of H-2\(^d\) stimulates rather than inactivates PLP1-specific T cells to sustain the severity of EAE. To test this premise, we sought to antagonize PLP1-specific T cells and determine whether disease exacerbation by agg Ig-PLP1 persists.

It has previously been shown that replacement of the TCR contact residues 144W and 147H with 144L and 147R of PLP1 generates a peptide designated PLP-LR that functions as an antagonist of PLP1-specific T cells (13). Also, PLP-LR was previously expressed on Ig and the resulting Ig-PLP-LR chimera antagonized PLP1-specific T cells effectively (8, 14). Herein, Ig-PLP-LR was used to antagonize PLP1-specific T cells to test for the effect of agg Ig-PLP1 on MOG-induced EAE. Accordingly, (SJL × C57BL/6)F\(_1\) mice were induced for EAE with 300 \(\mu\)g of MOG peptide and treated with 300 \(\mu\)g of agg Ig-PLP1 three times at 4-day intervals beginning at the onset of clinical symptoms with either 300 \(\mu\)g or agg Ig-PLP-LR alone or in combination with 300 \(\mu\)g of agg Ig-PLP-LR antagonist. The mice were bled via tail veins 4 days after the last agg Ig-PLP1 injection. A group of mice induced for MOG/EAE but treated with PBS instead of agg Ig-PLP1 was included for comparison purposes.

**FIGURE 7.** Antagonism of PLP1-specific T cells by Ig-PLP-LR nullifies agg Ig-PLP1-mediated exacerbation of MOG-induced EAE. A. Groups of 6- to 8-wk-old (SJL × C57BL/6)F\(_1\) mice were induced for EAE with 300 \(\mu\)g of MOG peptide and treated three times every 4 days beginning at the onset of clinical signs with either 300 \(\mu\)g of agg Ig-PLP1 alone or in combination with 300 \(\mu\)g of agg Ig-PLP-LR antagonist. The mice were monitored daily and assessed for clinical scores throughout the observation period. B. Five- to 7-wk-old (SJL × C57BL/6)F\(_1\) mice were given 300 \(\mu\)g of soluble Ig-PLP-LR i.p. at days –10, –6, and –3 before disease induction. These mice along with a group of 6- to 8-wk-old untreated (SJL × C57BL/6)F\(_1\) mice were then induced for EAE with 300 \(\mu\)g of MOG peptide. At the onset of clinical symptoms, both groups of mice were treated three times, 4 days apart with 300 \(\mu\)g of agg Ig-PLP1 and monitored for clinical signs of EAE daily until day 40 post disease induction.

**FIGURE 8.** agg Ig-PLP1 treatment diminishes anti-MOG Abs. Groups of 6- to 8-wk-old (SJL × C57BL/6)F\(_1\) mice were induced for EAE with 300 \(\mu\)g of MOG peptide and treated with 300 \(\mu\)g of agg Ig-PLP1 three times at 4-day intervals beginning at the onset (loss of tail tone) of clinical signs of EAE. The mice were bled via tail veins 4 days after the last agg Ig-PLP1 injection. A group of mice induced for MOG/EAE but treated with PBS instead of agg Ig-PLP1 was included for comparison purposes. α-bearing (A) and total (B) MOG Abs from the untreated (Nil) and agg Ig-PLP1-treated (Ig-PLP1) mice were measured by ELISA using the Southern Biotechnology Associates kit as described in Materials and Methods. Isotyping for γ1 (C), γ2a (D), γ2b (E), γ3 (F), α (G), and μ (H) class and subclass of anti-MOG Ab were also determined by ELISA using the Southern Biotechnology Associates kit as described in Materials and Methods. Serial serum dilutions were used in these analyses and the results shown were those obtained with 1/500 dilution. Each bar represents the mean ± SD of triplicates from five mice. * Significant difference relative to control untreated mice as analyzed by Student’s t test.
represents the mean mice were treated with agg Ig-PLP1 as in measured by ELISA as described in Materials and Methods.

FIGURE 9. agg Ig-PLP1 treatment exacerbates MOG/EAE through induction of IL-5 production by PLP1-specific T cells. In A, groups of 6- to 8-wk-old (SJL × C57BL/6)F1 mice were induced for EAE with 300 μg of MOG peptide and treated with 300 μg of either agg Ig-PLP1 or agg Ig-MOG three times at 4-day intervals beginning at the onset (loss of tail tone) of clinical signs of EAE. A group of mice that was induced for MOG/EAE but was not treated with any chimera (Nil) was included as control. The mice were sacrificed 4 days after the last treatment with the chimeras and their LN cells (5 × 105 cells/well) were stimulated in vitro with PLP1 (15 μg/ml), MOG (30 μg/ml), or HA (30 μg/ml) and cytokine production was measured by ELISA as described in Materials and Methods. Each bar represents the mean ± SD of triplicate wells. In B, groups of MOG/EAE mice were treated with agg Ig-PLP1 as in A and received an injection of 500 μg of anti-IL-5 Ab (TRFK-5) or rat IgG control along with the second agg Ig-PLP1 treatment. The mice were then monitored daily for clinical signs of EAE. Each point represents the mean score of eight mice. *, Values of p < 0.05 as determined by two-way ANOVA described in Materials and Methods.

of clinical signs declined significantly earlier and the disease resolved by day 30 while those not pretreated with agg Ig-PLP-LR did not recover for the 40-day disease monitoring period. Altogether, these results indicate that PLP1-specific T cells need to be functional to contribute to disease exacerbation upon treatment of MOG-induced EAE with agg Ig-PLP1.

TREATMENT OF MOG EAE WITH agg Ig-PLP1 INDUCES IL-5 THAT INHIBITS ANTI-MOG AB RESPONSE AND SUSTAINS EXACERBATION OF DISEASE IN F1 MICE

The results presented above indicate that MOG-specific T cells are activated during treatment with agg Ig-PLP1 but not required for exacerbation of disease while PLP1-specific T cells are. The question then is how PLP1-specific T cells contribute to exacerbation of disease.

MOG EAE usually involves Abs that contribute to the pathology of EAE (23, 24). If treatment with agg Ig-PLP1 leads to an increase in the production of MOG-specific Abs, it would result in exacerbation of disease. To address this postulate, F1 mice were immunized with MOG peptide, treated with agg Ig-PLP1 and their sera were tested for the presence of anti-MOG Abs. The results illustrated in Fig. 8 show, surprisingly, a significant reduction rather than increase in κ bearing (Fig. 8A), as well as total, anti-MOG Abs (Fig. 8B). Furthermore, the inhibition is more prevalent for both IgG2a and IgG2b isotypes (Fig. 8, D and E) rather than IgG1, IgG3, IgA, IgM classes of Ig (Fig. 8, C and F–H).

Because PLP1-specific T cells seem to be involved in agg Ig-PLP1-mediated exacerbation of MOG EAE and cytokines can control the magnitude and class of Ab responses, we sought to determine whether PLP1-specific cells and their cytokines interfere with the production of anti-MOG Abs. In an initial experiment, we immunized F1 mice with MOG peptide, treated the animals with agg Ig-PLP1 or control Ig-MOG that did not exacerbate the disease, and tested the T cells for differential cytokine expression. Among many cytokines tested including IFN-γ, IL-4, TGF-β, IL-17, only IL-5 was differentially produced in agg Ig-PLP1- vs agg Ig-MOG-treated mice (Fig. 9). Indeed, IL-5 is significantly increased upon stimulation of LN cells with PLP1 but not MOG peptide in the agg Ig-PLP1-treated mice (Fig. 9A). However, in the mice treated with agg Ig-MOG, neither PLP1 nor MOG peptide stimulation was able to induce significant IL-5 production. In fact, IL-5 in these mice was at background level relative to mice that were not treated with any chimera. These results suggest that IL-5 produced by PLP1-specific T cells may be responsible for disease exacerbation in agg Ig-PLP1-treated MOG EAE. To test this premise, F1 mice were induced for EAE with MOG peptide, treated with agg Ig-PLP1 with either anti-IL-5 Ab or rat IgG control, and the severity of disease was monitored. Fig. 9B shows indeed that neutralization of IL-5 in vivo inhibits disease exacerbation and the maximal severity of paralysis was reduced from 2.3 ± 0.2 to 1.3 ± 0.4. These results indicate that IL-5 from PLP1-specific T cells is responsible for disease exacerbation in agg
Ig-PLP1-treated animals. Because the mice with exacerbated disease had significant IL-5, displayed diminished production of MOG Abs, and neutralization of IL-5 reduced disease severity, we suspected that IL-5 exacerbates disease by suppression of MOG Abs. If this is the case, then neutralization of IL-5 which inhibited disease severity should restore production of MOG Abs. Indeed, Fig. 10 shows that mice recipient of anti-IL-5 Ab during treatment with agg Ig-PLP1 restored production of total anti-MOG Abs (Fig. 10A). Moreover, IgG2a and IgG2b isotypes which were reduced by treatment with agg Ig-PLP1 were restored by neutralization of IL-5 (Fig. 10, C and D) but IgG1 subclass which was not affected by treatment with Ig-PLP1 was not increased by neutralization of IL-5 (Fig. 10B). These results indicate that IL-5 from PLP1-specific T cells diminishes MOG Abs and sustains exacerbation of MOG EAE in F1 mice.

Discussion

In previous studies, we showed that both agg Ig-PLP1 and agg Ig-MOG effectively suppress EAE by a “dual modal” mechanism involving minimal costimulation and IL-10 bystander suppression (4, 5, 8). The approach is attractive because it targets both Ag-specific T cells, as well as nearby autoreactive T cells of unrelated specificities (4, 5, 8). These reports examined the efficacy of the Ig delivery system for treatment of autoimmunity in nonpolymorphic inbred mouse strains. However, these animals do not nearly represent the genetic diversity seen in humans. In this study, we examined whether the Ig approach would be able to exert similar suppressive effectiveness in a polymorphic setting with relevance to humans. Accordingly, F1 mice were generated by breeding SJL/J to B10.PL or C57BL/6 and agg Ig-PLP1 as well as agg Ig-MOG were tested for suppression of CNS homogenate-induced EAE where the full spectrum of epitopes restricted to both parental haplotypes is play at the chimeras would have to modulate diverse T cell specificities to ameliorate the disease. Both agg Ig-PLP1 and agg Ig-MOG were quite effective at reversing EAE induced with CNS homogenate in the (SJL/J × B10.PL)F1, mice (Fig. 1). Unexpectedly, however, when the same experiment was performed in the (SJL/J × C57BL/6)F1 strain, neither Ig chimera was able to ameliorate disease. This was intriguing and prompted investigation at the single epitope level where EAE is induced by an epitope restricted to the haplotype of one parent and the treatment is made with a chimera carrying a peptide with the same parental restriction or a peptide restricted to the other parent (in trans treatment). Accordingly, groups of (SJL × C57BL/6)F1 mice were induced for EAE with PLP1 or MOG peptide and the animals were treated with either agg Ig-PLP1 or agg Ig-MOG. It was observed that both chimeras were able to ameliorate PLP1-induced EAE (Fig. 2 and Table I). However, in the animals induced for EAE with MOG peptide, Ig-MOG reduced the severity of disease while in trans treatment with Ig-PLP1 exacerbated the clinical signs of MOG/EAE, where the initial phase of disease was more pronounced and the resolution was delayed. Moreover, prolonged in trans treatment with agg Ig-PLP1 did not change the disease exacerbation pattern (Fig. 3) suggesting delayed spreading to PLP1 epitope after completion of the three-injection regimen was not the mechanism responsible for disease exacerbation. In fact, MOG-specific IFN-γ production was evident during exacerbation of disease by agg Ig-PLP1 treatment (Fig. 4). Also, spreading to epitopes other than PLP1 and MOG was excluded because when Ig-PLP1 was accompanied with Ig-MBP3 or Ig-PLP2 exacerbation of EAE persisted (Fig. 6). This was intriguing and led us to suspect involvement of CD8 T cells. Indeed, there are reports in the literature indicating that MOG 35–55 encompasses an epitope recognized by CD8 T cells in the C57BL/6 mouse (18, 19). Because aggregation of the Igs induces the APCs to produce IL-10, which is a growth factor for CD8 T cells (17), and we found that the anti-MOG response is maintained in agg Ig-PLP1-treated mice compared with untreated, there was a possibility that agg Ig-PLP1 was sustaining activation and expansion of MOG-specific CD8 T cells. However, this postulate proved incorrect as Ag-specific IFN-γ could be inhibited only in the presence of anti-CD4 Ab indicating that CD8 T cells play little or no role in agg Ig-PLP1-mediated exacerbation of MOG-induced EAE (Fig. 5).

In the face of this dilemma, we were left with the possibility that agg Ig-PLP1 may be activating, rather than tolerizing, PLP1-specific T cells, leading to exacerbation of disease. To examine this possibility, agg Ig-PLP1 was combined in a treatment with Ig-PLP-LR, an Ig carrying a PLP1 antagonist (8). The results indicated that agg Ig-PLP-LR reduced the severity of disease during treatment with agg Ig-PLP1 (Fig. 7). Furthermore, when antagonism was conducted before disease induction with MOG peptide, treatment with agg Ig-PLP1 exacerbation of disease was nullified (Fig. 7). This indicates that PLP1-specific T cells are required for exacerbation of disease (Fig. 7). The question then is how these T cells aggravate MOG/EAE. Cytokine screening analysis indicated that in the MOG/EAE mice treated with agg Ig-PLP1 there was production of IL-5 by PLP1-specific T cells that was not observed in untreated or Ig-MOG-treated mice (Fig. 9). Given that IL-5 is a Th2 cytokine usually associated with modulation of autoimmunity, we concluded that it might be indirectly involved in the exacerbation of disease. Knowing that MOG/EAE involves Abs (23, 24), we suspected that IL-5 may be interfering with such Ab responses to aggravate the disease. Surprisingly, however, treatment with agg Ig-PLP1 reduced MOG Ab responses and more specifically the IgG2a and IgG2b isotypes (Fig. 8). Furthermore, neutralization of endogenous IL-5 modulated the disease (Fig. 9) and restored Ab responses with reincrease of IgG2a and IgG2b anti-MOG isotypes to significant serum levels (Fig. 10). Because IL-5-deficient mice manifest signs of EAE similar to wild-type mice (25), it is possible that other cytokine products of Th2 or T regulatory cells contribute to the Ig-PLP1-mediated aggravation of EAE. The fact that disease progression occurs when serum anti-MOG Ab titers diminish and the severity is reduced when the Ab titer reincreases (by neutralization of IL-5) suggests that the Abs may play a protective role. It has previously been shown that MOG-specific Abs directed against conformational epitopes are pathogenic while those recognizing linear epitopes have no demyelinating effects (26, 27). In this study, because the Abs are induced and detected by the MOG 35–55 peptide, they represent anti-linear epitope Abs that seem to play a protective rather than pathogenic role. Given that MOG is restricted to C57BL/6 haplotype and that this strain is unable to develop pathogenic Abs to conformational MOG epitopes (28), it is likely that the linear epitope-specific Abs contribute protective rather than demyelinating functions. The conclusion that can be drawn from these studies suggests that T cell tolerance in the context of genetic polymorphism could nullify protective humoral responses and aggravate rather than ameliorate autoimmunity.

Overall, Ag-specific therapies are ideally more suitable for treatment of autoimmune disorders than non-Ag-based therapies, presumably because they affect the specific cells responsible for the pathogenesis of the disease. However, complex polymorphisms which could result in unbalanced MHC expression (29) need to be taken into consideration to devise effective Ag-specific therapy against the disease.

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References