The beneficial clinical effects of teriflunomide in experimental autoimmune myasthenia gravis and the investigation of the possible immunological mechanisms

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Abstract

Myasthenia Gravis (MG) is an autoantibody-mediated autoimmune disease characterized by skeletal muscle weakness exacerbated with exercise. There is a need for novel drugs effective in refractory MG patients. We aimed to test the potential of teriflunomide, an immunomodulatory drug currently used in rheumatoid arthritis and multiple sclerosis treatment, in a murine experimental autoimmune myasthenia gravis (EAMG) model. EAMG was induced by immunizations with recombinant acetylcholine receptor (AChR). Teriflunomide treatment (10 mg/kg/day, intraperitoneal) was initiated to one group of mice (n=21) following the third immunization and continued for five weeks. The disease control group (n=19) did not receive medication. Naïve mice (n=10) received only mock immunization. In addition to the clinical scorings, the numbers of B cells and T cells, and cytokine profiles of T cells were examined by flow cytometry. Anti-AChR specific antibodies in the peripheral blood serum were quantified by ELISA. Teriflunomide significantly reduced clinical disease scores and the absolute numbers of CD4+ T cells and some of their cytokine producing subgroups (IFN-γ, IL-2, IL-17A, GM-CSF) in the spleen and the lymph nodes. The thymic CD4+ T cells were also significantly reduced. Teriflunomide mostly spared CD8+ T cells' numbers and cytokine production, while reducing CD138+CD19+ lambda+ plasma B cells' absolute numbers and CD138 mean fluorescent intensities, probably decreasing the number of IgG secreting more mature plasma cells. It also led to some selective changes in the measurements of anti-AChR specific antibodies in the serum. Our results showed that teriflunomide may be beneficial in the treatment of MG in humans.

1. Introduction

Myasthenia Gravis (MG) is an antibody-mediated autoimmune disease characterized by skeletal muscle weakness worsened by exercise. Although a rare disease, MG impairs the daily activities of patients, and if untreated, in up to 20% percent of the cases, leads to myasthenic crisis which is characterized by exacerbated muscle weakness, respiratory failure eventually requiring intubation and mechanical ventilation (Fichtner et al., 2020; Howard, 2018; Wang and Yan, 2017).

MG results from antibody-mediated destruction of the postsynaptic membrane at the neuromuscular junctions (NMJ) and has several subtypes depending on the antigen specificity of the autoantibodies (Fichtner et al., 2020). In about 85% of the patients, nicotinic acetylcholine receptor (n-AChR) specific autoantibodies are found (Vincent, 2002). Muscle- specific kinase (MuSK) (6%) or lipoprotein receptor-related protein (LPR4) (4%) specific autoantibodies have been shown in the remainder of the patients (Higuchi et al., 2011; Hoch et al., 2001; Zisimopoulou et al., 2014). About 5% of the patients are classified as seronegative MG and no known autoantibody or antigen has been defined in those patients to this date (Carr et al., 2010; Higuchi et al., 2011; Hoch et al., 2001; Zisimopoulou et al., 2014). The majority of the AChR autoantibodies are produced by long-lived (mature) plasma cells and are of IgG1 isotype, thus can mediate complement-mediated damage of NMJs. MuSK autoantibodies, however, are mainly of IgG4 isotype and produced by short-lived plasmablasts (McConville et al., 2004; Stathopoulos et al., 2017).

The immunopathogenesis of MG involves innate and adaptive immune cells (Wang and Yan, 2017). Initial antigen presentation to T cells may occur peripherally, or in the thymus due to various stimuli including infections. Elevated or activated CD4 + T helper cells subsets, including Th1, Th17 and Tfh have been reported in MG patients suggesting their involvement at various stages of disease pathogenesis (Wang and Yan, 2017). The thymus appears to be particularly involved in MG pathogenesis not just as an organ for T cell generation but acting as a site for the expression of AChR alpha subunit and being hyperplastic in 70% of AChR MG patients and harboring autoreactive B cells and T cells (Filosso et al., 2013).

Currently, long- term treatment of MG is based on a combined therapy which includes symptomatic treatment with acetylcholine esterase inhibitors and immunosuppressants corticosteroids, azathioprine mycophenolate mofetil, as well as rituximab which has been widely used more recently (Silvestri and Wolfe, 2014; Sonkar et al., 2017). Additionally, in trials, C5 inhibitor eculizumab improved generalized MG (gMG) with AChR autoantibodies, or refractory gMG, and it is currently an emerging therapy in MG. However, the duration of its use as a treatment, its long- term tolerability, its efficiency and cost of effectiveness are not clear yet. (Dhillon, 2018). Despite various treatment modalities, some patients experience recurrent myasthenic crises with worsened muscle weakness. Additionally, long term use of currently available immunosuppressant drugs has important side effects. Thus, there is a need for novel treatment options, with fewer side effects, suitable for long- term use and effective in refractory cases.

Teriflunomide is an immunomodulatory drug approved by FDA and EC for use in relapsing-remitting multiple sclerosis (RRMS) in 2012 and 2013, respectively. Leflunomide, which is actively used in rheumatoid arthritis treatment since 2008, is structurally similar and is converted (70%) to teriflunomide *in vivo*. Thus, *in vivo* activity of leflunomide is thought to be largely that of teriflunomide. Teriflunomide inhibits pyrimidine *de novo* synthesis by reversibly inhibiting the mitochondrial enzyme dehydroorotate dehydrogenase (DHO-DH) at concentrations below 100μM. Therefore, teriflunomide blocks DNA synthesis, and in turn, the proliferation of rapidly dividing cells such as B and T cells by reducing cellular pyrimidine pool. At higher concentrations, teriflunomide has been shown to inhibit purine synthesis as well, thereby inhibiting ATP-dependent cellular processes. Studies in mice have shown that leflunomide was effective in reducing T-dependent and independent antibody responses. A more recent study showed that teriflunomide is beneficial in the MuSK-induced EAMG model (Yilmaz et al., 2021).

In the current study we aimed to test the therapeutic potential of teriflunomide in experimental autoimmune MG (EAMG) induced by AChR immunizations in mice. Teriflunomide was given to a group of EAMG mice after the disease induction and afterwards the disease clinical scores, the B and T cell immune responses, and the autoantibody productions were monitored over time. Our results revealed that teriflunomide may alleviate EAMG disease severity by reducing CD4 + T cell and subsequent B cell responses in a preclinical MG model.

2. Methods

2.1. Mice

Specific pathogen- free C57BL6 female mice were purchased from Kobay Ltd. (Ankara, Turkey) and housed at Erciyes University, Experimental Research and Application Center (DEKAM) vivarium after 14 days of quarantine. The mice were fed regular chow diet. The experiments were performed on 8-week old mice. The study was approved by Erciyes University animal studies institutional review board (#19/139). All the experiments were conducted according to relevant guidelines and regulations. The mice were randomly allocated into three groups: i) Experimental autoimmune myasthenia group which would take teriflunomide (EAMG + TF), ii) Experimental autoimmune myasthenia group (EAMG), and the iii) Healthy control group.

2.2. Induction of experimental autoimmune myasthenia gravis (EAMG)

Torpedo californica AChR (Acetylcholine Receptor) protein was purchased from Chinapeptides (Wujiang Scientific Innovation Park, China). The AChR protein was expressed in *E.coli*. Refolding was performed in NTAU-X buffer: 0.15 M NaCl, X mM imidazole, PBS, 8M Urea, pH7.2. The sequence is as follows: SEHETRLVAN LLENYNKVIR PVEHHTHFVD ITVGLQLIQL ISVDEVNQIV ETNVRLRQQW IDVRLRWNPA DYGGIKKIRL PSDDVWLPDL VLYNNADGDF AIVHMTKLLL DYTGKIMWTP PAIFKSYCEI IVTHFPFDQQ NCTMKLGIWT YDGTKVSISP ESDRPDLSTF MESGEWVMKD YRGWKHWVYY TCCPDTPY LD ITYHFIMQRI. Equal volume of complete Freund's adjuvant (CFA) and peptide diluted in phosphate buffered saline (PBS) (0.3 mg/mL) were emulsified using a stopcock and 5 cc syringes. Mice were immunized at 4 different spots with (50 µl/injection, and a total of 200 µl per immunization) on the lower flanks, both left and right, for the 1st immunization; at higher flanks for the 2nd immunization; and at the inguinal and lower flanks for the 3rd immunization. The mice were immunized on days 0, 21 and 48 and sacrificed on day 84 (12th week) of immunization. Twenty one of AChR-immunized mice (EAMG + TF group) received teriflunomide (TF) starting with the day 51, when 60% of the modeled mice (n:24) developed clinical symptoms of the disease. Teriflunomide was given intraperitoneally following the third AChR immunization until the mice were sacrificed at (10 mg/mL, daily) dose for 33 days. The EAMG group (AChR-immunized) received PBS instead of teriflunomide. The mice in the healthy control group (n = 11), were only immunized with CFA-PBS emulsion (without AChR). The experimental groups and the study design are shown in Fig. 1.

2.3. The clinical tests and the scoring

The clinical evaluations of the mice were carried out in a double blind fashion regularly with the start of the experiment in DEKAM. The weight and general condition of the mice were evaluated daily. Strength and fatiguability evaluations based on observation and examination

(Ulusoy et al., 2017) were accomplished every three days until the second dose of AChR immunization, and daily from the second dose immunization until the end of the experiment.

In addition to these assessments, the post-exercise inverted grid test (Yilmaz et al., 2021) was performed to monitor fatiguability. The clinical grade scorings of the mice were noted on a weekly base: Grade 1, normal muscle strength and no muscle weakness, even after exercise; grade 1, normal at rest but weak after exercise, with chin on the floor and inability to raise head, hunched back, and reduced mobility; grade 2, grade 1 weakness at rest; grade 3, severe weakness, dehydrated and paralyzed (quadriplegic), loss of significant weight; and grade 4, found dead in the cage (Tuzun et al., 2015). These scores were treated as the status of the mice at the end of the week, with the help of the close evaluations during the week.

The clinical assessments of the animal groups were parameterized as disease incidence, disease clinical score, and the ratio of actual body weights of animals to their weights at baseline, and comparisons of the groups were based on these parameters. In disease incidence assessments, mice were required to have at least a grade 1 clinical score to be considered as myasthenic.

2.4. The enzyme linked immunosorbent assay (ELISA)

Fifty µl of blood was collected from the facial veins of mice on day − 1, week 7 and 12. The serum was taken after centrifugation at 6000 g for 10 minutes and frozen at -80. The samples were thawed and diluted 1000 times and used for ELISA. The protocol by Yilmaz et al. was followed (Yilmaz et al., 2021). Briefly, AChR protein was diluted in coating buffer and the 96-well ELISA plate was incubated overnight at 4°C. The excess protein was washed with Wash Buffer (PBS 0.05% Tween). The plates were blocked with 5% FBS in PBS for 2 h and afterwards washed 3 times with Wash Buffer. The serum was added and incubated for 2 hours at room temperature. After 3 washes with Wash buffer anti-IgG1, IgG2b, IgG3 and IgM were added with 5000-fold dilution. The ELISA experiments were performed two times.

2.5. The surface and intracellular cytokine staining

The splenocytes, the lymph nodes and the thymuses were harvested at the twelfth week post-immunization into collection tubes in 1 mL PBS. The tissues were physically macerated over a 70 µm strainer and the single-cell suspension of leucocytes was washed once, by centrifugation at 300 g, 5 min. Splenocytes were treated with RBC Lysis Buffer (Gibco) and washed once, by centrifugation at 300 g, 5 min. Splenocytes (after erythrocyte removal), thymocytes or lymph node leucocytes were resuspended in complete media (RPMI 1640 supplemented with L-Glutamine, anti-anti and 10% FBS (fetal bovine serum), counted with Trypan Blue and used for experiments. For surface staining, ($1x10^5$) were stained directly without further stimulation. For intracellular cytokine staining, from each mouse, ($1x10^5$) cells were seeded into U-bottom 96 well-plates in 200 µl volume with phorbol 12-myristate 13-acetate (PMA) (Sigma, 79346, 50 ng/mL) and Ionomycin (Sigma, I3909, 1 µg/m). All wells also received Golgi Plug (BD, 1µL/mL). After 5 hours of culture at 37°C and 0.05 CO_2, cells were collected spun for 3 min at 400 g at 4°C. Pellet was surface stained for CD4 (clone: SK3), CD3 (clone: HIT3a) first for 30 min in Staining Buffer (PBS with 2% FBS) and washed twice with Staining Buffer (400 g centrifugation for 3 min). Then, the cells were stained for IL-22 (clone: 2G12A41), IL-17A (clone: BL168), IFN-□ (clone: 4S.B3), GM-CSF

(clone: BVD2-21C11), IL-2 (clone: MQ1-17H12) using BD Cytofix/Cytoperm™ Fixation/ Permeabilization *Kit (554714)* according to manufacturer's instructions. Antibodies were purchased from SONY.

3. Statistical Analyses

GraphPad Prism 6 program was used for the statistics and for the generation of the graphs. One-way analyses of variance (ANOVA) or Kruskall Wallis test was used appropriately. Tukey's multiple comparison test was applied to correct for statistical hypothesis testing after ANOVA. Dunn's correction was applied after Kruskall Wallis test for the same purpose. The 7th and 12th weeks' serum immunoglobulin levels of each individual mouse in the study groups were compared with each other by using paired t test. This test was repeated for each myasthenic experimental group (EAMG + TF and EAMG). P-value < 0.05 was regarded as significant.

4. Results

4.1 Teriflunomide ameliorates the disease scores and the incidence in EAMG model induced by recombinant Torpedo AChR protein immunization

To test the therapeutic potential of teriflunomide on EAMG, the disease was induced by three consecutive recombinant *Torpedo* AChR protein immunizations on the days 0, 21 and 48. Naïve unimmunized (control), immunized (EAMG) and teriflunomide-receiving immunized (EAMG + TF) groups were compared in detail with respect to weight gain, incidence and EAMG clinical scores (Fig. 2a-c). Following the third AChR immunization, teriflunomide 10 mg/kg was given intraperitoneally daily for 33 days until the mice were sacrificed. The EAMG group received PBS.

The body weight gain slowed down in the EAMG mice compared with healthy controls at 8th weeks after first immunization (Fig. 2a). At 11 and 12 weeks, weight gain in EAMG + TF was further reduced compared to EAMG group. However at this dose teriflunomide did not cause any weight loss but rather reduced the rate of weight gain. Teriflunomide treatment reduced disease incidence at 12th week significantly (Fig. 1b), and ameliorated disease scores significantly starting at 10th week of EAMG until the end of experiment (Fig. 2c). Thus, teriflunomide treatment appears to improve clinical disease scores in AChR-specific EAMG.

4.2. Teriflunomide inhibits CD4 + T cell responses in the lymph node, spleen and the thymus

Since teriflunomide blocks DNA synthesis in rapidly proliferating T cells (Bar-Or et al., 2014; Loffler et al., 2004; Ruckemann et al., 1998), we explored its impact on T cell numbers and cytokine production in the spleen, draining lymph nodes (inguinal) and the thymus of the three mice groups. The spleen, thymus

and the lymph nodes were collected at the end of the 12th week of EAMG induction. The absolute number of splenic CD3 + CD4 + T cells were comparable between the naïve unimmunized (control), immunized (EAMG) or immunized and teriflunomide-receiving (EAMG + TF) groups (Fig. 3a). However, splenic IL-2+, IL-17A+, IFN-γ+, IL-22+, IL-10 + CD3 + CD4 + T cells were significantly reduced in the EAMG + TF group compared with EAMG (Fig. 3c-h). The percentages of the cytokine- producing cells among splenic CD3 + CD4 + T cells were significant only for IFN-γ + cells but not others (Fig. 3b). However, the mean fluorescence intensity (MFI) of IFN-γ was comparable between EAMG + TF and EAMG groups (Fig. 4c). These results suggested that the reduction in the cytokine-producing splenic CD4 + T cells was due to reduced numbers of cells rather than a decrease in gene expression or protein synthesis.

In contrast to the spleen, the draining lymph node had significantly fewer CD3 + CD4 + helper T cells in the EAMG + TF mice compared with the EAMG group (Fig. 5a). Additionally, the absolute number of IFN-γ+, GM-CSF+, IL-22+, IL-10 + CD3 + CD4 + T cells were significantly reduced in the lymph nodes of the EAMG + TF group compared with the EAMG mice group (Fig. 5b-h). The percentage of IFN-γ + CD4 + T cells was also reduced significantly (Fig. 6b). However, similar to spleen, MFI values of IFN-γ were comparable between EAMG + TF and EAMG groups (Fig. 6c), suggesting that the reduction in the cytokine-producing splenic CD4 + T cells were due to reduced numbers of cells rather than a decrease in gene expression or protein synthesis.

Unlike CD4 + CD3 + T cells, teriflunomide had no impact on total splenic CD8 + CD3 + T cells numbers or the number of their IL-2, IL-17A, IFN-γ, GM-CSF, IL-22, IL-10, TNF-α producing subsets compared with EAMG groups (Fig. 3i-p). Similarly, CD8 + CD3 + T cells in the lymph nodes or their cytokine- producing subsets were comparable between EAMG + TF and EAMG groups (Fig. 4i-p). The gating strategies and the percentage values of T lymphocytes in the spleen and lymph nodes are shown in Figs. 6 and 7.

Thymic hyperplasia is a critical component of MG pathogenesis in 70% of MG patients with AChR autoantibodies. The hyperplastic thymus has autoreactive B and T cells. Although thymic involvement in EAMG mice was considered to be minimal (Mantegazza 2016), we also assessed the impact of teriflunomide on thymic T cell output in the AChR immunized mice (Fig. 7). Our data revealed a significant increase in the absolute number of thymic CD3 + CD4 + T cells in EAMG mice compared with the naïve control group (Fig. 6c). Teriflunomide treatment after disease onset significantly reduced the absolute number of CD3 + CD4 + T cells. Although the reduction in CD8 + T cells was not statistically significant, CD40L expressing CD8 + T cell (activated CD8 + T cell) numbers were significantly reduced after teriflunomide treatment (Fig. 7h). These results collectively suggest that in the EAMG context induced by AChR immunizations, teriflunomide greatly reduces both, CD4 + T cell expansion and CD8 + T cell activation.

4.3. Teriflunomide reduces the CD138 expression and the lambda positive plasma cells

To further assess the impact of teriflunomide on B cell or plasma cell numbers, spleen and lymph nodes of naïve unimmunized (healthy control), immunized (EAMG) or immunized and teriflunomide-receiving

(EAMG + TF) groups were stained for CD19, CD138, IgG kappa or IgG lambda. The gating strategies of lymphocytes in the spleen and lymph nodes is shown in Fig. 8.

Total B cells were gated as CD19 + cells (Fig. 8) and were unaffected with teriflunomide treatment as shown by comparable CD19 + B cell numbers both in the spleen and the lymph nodes (Fig. 9a and f). Similarly, plasma B cell (marked as $CD19^{+/low}$ CD138+) absolute numbers were comparable across all the groups (Fig. 9b and g). However the absolute number of IgG lambda + plasma cells in the spleen was significantly reduced in the EAMG + TF group as compared to the EAMG group (Fig. 9c). Furthermore, CD138 expression by $CD19^{+/low}$ cells reflected by MFI CD138 was significantly reduced in the EAMG + TF group in comparison to EAMG group (Fig. 9e and h). These results suggest that teriflunomide treatment after the onset of EAMG, may selectively reduce IgG lambda + plasma cells and may interfere with the functions of plasma cells by reducing the surface CD138 expression, probably indicating a decrease in the number of more mature plasma cells.

4.4. Teriflunomide changes the amount of serum anti-AChR antibodies in a selective way

Because both human MG and EAMG are antibody-mediated diseases, we directly measured the impact of teriflunomide on levels of anti-AChR antibodies (Fig. 10). When basal, after the disease induction pre- and post-treatment (at the 7th and 12th week: on the days of 49 and 83) levels of anti-AChR immunoglobulins (IgG1, IgG2b, IgG3, IgM) were evaluated, all four AChR specific immunoglobulin levels were elevated at 7th week in the disease groups and were further augmented following the last immunization by 12th week. The teriflunomide injections were initiated on the day 51st post- immunization and continued until mice were sacrificed. At the end of the treatments, at the 12th week, there was no significant difference between the EAMG and EAMG + TF groups regarding AChR-specific IgG1, IgG2 or IgG3 (Fig. 10). However, the AChR-specific IgM levels were significantly higher by the 12th week in the EAMG + TF mice compared with the EAMG group ($p<0.05$) (Fig. 10d).

When immunoglobulin levels of each mouse at 7th (in the pre-treatment period) and 12th week (in the post-treatment period) were together considered within each disease model groups by using paired t-tests, all immunoglobulins seemed to increase at the significance level of $p < 0.001$ in both EAMG+TF and EAMG groups after treatment. Only IgG1 increase in the EAMG + TF mice group, showed a lower significance level ($p< 0.05$) compared to other significance levels ($p < 0.001$) probably reflecting a subtle reduction in the IgG1 levels (Fig. 11). These data collectively support that the EAMG model in the study has been successfully implemented and that teriflunomide administration after disease onset may affect IgG antibody isotypes in a selective way.

5. Discussion

In the current study, the therapeutic effects of the cytostatic and immunomodulatory drug teriflunomide were evaluated in a mouse model of MG induced by AChR immunizations. Our results revealed that

teriflunomide treatment after disease onset decreased the incidence and clinical disease scores of murine EAMG. Teriflunomide improved EAMG symptoms by reducing the absolute CD4+ T cell numbers and cytokine production, the number of certain plasma cell subsets and by decreasing long lived mature plasma cell expression (CD138 expression) and causing a subtle decrease in IgG1 type anti-AChR in comparison to other types of immunoglobulins. To our knowledge, this is the first study showing a clinical and immunological benefit of teriflunomide use after disease onset in an anti-AChR-mediated model of EAMG in mice. Yilmaz et al. have recently tested therapeutic administration of teriflunomide in MuSK mediated-EAMG and showed clinical benefit similar to the results presented herein (Yilmaz et al., 2021). AChR-specific autoantibody- mediated pathology account for 85% of MG cases in humans; while MuSK autoantibody-mediated MG accounts for 6% of the MG patient population (Fichtner et al., 2020; Wang and Yan, 2017). Therefore, the results of the current report have key implications for MG patients, and supports the use of teriflunomide as a therapeutic treatment.

In our hands, the clinical EAMG disease was first observed in some mice about the fourth week (ten days after the second immunization). Disease incidence and mean disease score reached a statistically significant level compared to the healthy control group at the sixth week after the first immunization (three weeks after the second AChR injection), which is consistent with the literature (Mantegazza et al., 2016; Shigemoto et al., 2015; Yilmaz et al., 2021). In some studies, the standards of mimicking MG treatment after disease onset is defined as the time when ≥ 60 % of mice develop clinical symptoms of the disease (Tuzun et al.,2015). In our experiment, this criterion was fulfilled three days after the third immunization and the treatment was started at this time. Teriflunomide treatment after disease onset reduced the anti-AChR antibody mediated EAMG incidence at 12^{th} week, and the mean clinical MG scores starting at 10^{th} week till the end of the experiment compared with untreated EAMG mice. The data indicate that teriflunomide's clinical effects are detectable after three weeks of use. In Yilmaz et al.'s MuSK mediated EAMG model, the beneficial effect of teriflunomide was also detectable starting by the 11^{th} week of disease induction, thus these independent studies corroborate each other's findings. Although EAMG dependent weight loss was ameliorated by teriflunomide treatment in the Yilmaz et al. study (Yilmaz et al., 2021). Our EAMG+TF group showed no weight loss, but reduced weight gain compared with the EAMG group. The difference between the two studies could be explained by differences in the doses of teriflunomide, which was higher in the current study (Committee for Medicinal Products for Human Use-CHMP- 2013).

The primary mechanism of action of teriflunomide is through inhibition of mitochondrial enzyme DHO-DH. This enzyme is highly expressed in activated lymphocytes, both T and B cells which rely on de novo pyrimidine synthesis to meet their increased nucleotide demands. Because it is not a nucleotide analogue, and the resting lymphocytes can use salvage pathways to meet their pyrimidine needs, teriflunomide appears to affect mostly activated lymphocytes (Bar-Or et al., 2014). *In vitro* proliferation assays revealed that teriflunomide can inhibit proliferation of CD4+, CD8+ T cells as well as B and NK cells without reducing their survival (Li et al., 2013). Others have shown that, *in vivo*, teriflunomide was

particularly effective in inhibiting the proliferation of T cells with high-affinity T cell receptors for the antigens (Posevitz et al., 2012).

In our experiments regarding the T cell compartment, the most notable changes were observed in CD4+ helper T cells with teriflunomide treatment. In the Yilmaz et al study, flow cytometric analyses of T cells (CD4+ or CD8+), B lymphocytes as well as natural killer (NK) cells revealed no significant difference between teriflunomide treated and untreated MuSK EAMG groups with respect to percentages of cells. However the changes in the absolute numbers of those cells have not been documented in that study (Yilmaz et al., 2021). Our experiments also did not reveal any reduction in the percentages/frequency of CD4+ T cells or their IL-2+, IL-17+, IL-22+, GM-CSF+ subsets between teriflunomide treated and untreated AChR EAMG groups. However, the absolute number of IFN-γ+, IL-17+, IL-2+, IL-22+ CD4+ T subsets as well as total CD4+ T cells were reduced significantly in the secondary lymphoid organs after teriflunomide treatment. The anti-proliferative effect of teriflunomide on CD4+ T cells was also evident in the thymus. Thus, helper T cells responses, particularly Th1, Th17 which were shown to play critical roles in the pathogenesis of EAMG, appeared to be inhibited by teriflunomide. This finding has been supported by the existing literature (Wang and Yan, 2017). When IFN-γ MFI values were examined, IFN-γ expression per cell was unaffected. These findings argue that teriflunomide, rather than inhibiting transcription/or translation events of these cytokine genes, acts by inhibiting proliferation of those T helper cell subsets. These data are in line with the human studies showing leukopenia/lymphopenia in MS patients who were treated with teriflunomide and the well-established negative impact of teriflunomide on highly proliferating T and B cells (Bar-Or et al., 2014; Confavreux et al., 2014; O'Connor et al., 2011). It is important to note that T and B cells' frequency/percentage measurements do not always reflect the changes in the absolute number of cells, and discrepancies between some reports most likely result from the lack of absolute number calculations.

In our experiments, teriflunomide did not reduce total CD8+ T cell numbers *in vivo* in EAMG mice, be it in lymph nodes, spleen or thymus. However, we observed a reduction in CD40L+ CD8+ T cell numbers (activated CD8+ T cells) in the thymus. Both CD4+ and CD8+ T cells are shown to be involved in the pathogenesis of EAMG in rats and mice, unlike human MG, which occurs predominantly through CD4+ T cell and B-cell mediated pathology (Wang and Yan, 2017; Zhang et al., 1996). Accordingly, genetic or antibody-mediated depletion of CD8+ or CD4+ T cells suppressed the disease (Zhang et al., 1996). It is unclear why teriflunomide has a more robust impact on preferentially CD4+ T cell compartment in the murine EAMG model. This could be related to differential reliance of CD4+ and CD8+ T cells on DHO-DH and requires further study. Although a recent study suggested that 12-month use of teriflunomide in relapsing- remitting MS patients selectively reduced CD8+ memory T cells, absolute numbers of cells have not been examined in that study (Tilly et al., 2021). Another report performed on seven multiple sclerosis (MS) patients by Gandoglia et al. showed a trend towards reduction in helper T cells after teriflunomide use (Gandoglia et al., 2017).

Both leflunomide and teriflunomide have been shown to inhibit B cell proliferation through inhibition of DHO-DH and other targets such as cyclin D3 and cyclin A expression (Ringshausen et al., 2008). In the

peripheral blood of teriflunomide receiving MS patients, absolute numbers of mature, regulatory or CD19+ total B cells were significantly reduced (Gandoglia et al., 2017). The only report investigating B cells in the EAMG mice model was Yilmaz et al.'s anti-MuSK mediated EAMG study. In that study, B cell percentages were found not to be significantly altered, while the absolute B cell counts were not examined (Yilmaz et al., 2021). Our data revealed that total CD19+ B cells as well as plasma cells ($CD19^{+/low}$ CD138) absolute numbers were not significantly altered by teriflunomide treatment. On the other hand, Lambda+ plasma cell absolute numbers were significantly reduced in the spleen of EAMG+TF mice compared with the untreated EAMG group. Additionally, CD138 expression (mean fluorescence intensity showing mean antigen expression), which is high in long lived mature plasma cells responsible for the production of IgG, were significantly reduced in the lymph nodes and the spleen suggesting that teriflunomide may result in significant changes in the plasma cell functions affecting antibody production in a selective way (Bortnick and Allman, 2013; Nutt et al., 2015).

In our experiments, teriflunomide treatment appears to increase IgM isotype, while it also causes a subtle decrease in IgG1 level. Although IgG1 levels were still comparable between EAMG and EAMG+TF mice groups in the 12-week post EAMG induction, IgG1 levels at 12^{th} week of disease induction was more significant in the EAMG group compared to EAMG+TF group when compared to their corresponding basal levels. These data argue that the drop in IgG1 levels EAMG+TF mice group is continuing, yet undetectable at this time point. Indeed, in the anti- MuSK mediated EAMG model of Yilmaz et al. serum IgG1 levels significantly reduced after teriflunomide treatment at the 14^{th} week of disease induction (Yilmaz et al., 2021). Besides reduced IgG deposition at the neuromuscular junctions was reported in that study, supporting our data. Yılmaz et al did not measure serum IgM levels in their study (Yilmaz et al., 2021).

When both studies of teriflunomide in EAMG are evaluated together, it can be reported that this drug may cause some selective changes in the antibody responses. Upon considering the basic mechanism of action of teriflunomide that is inhibiting the rapidly progressing T and B cells, to explain these selective changes in the antibody responses seems to be difficult. Through its basic mechanism of inhibitory action on DHO-DH, teriflunomide has been proposed to have direct suppressive effect on B cells, since it reduced proliferation and lipopolysaccharide stimulated Ig secretion of B cells (Siemasko et al., 1996). Siemasko et al. in their *in vivo* study found that leflunomide decreases both IgG and IgM secretion, in a manner that is mostly decreasing IgG levels (Siemasko et al., 1998). They also discovered that leflunomide has some DHO-DH independent functions including tyrosine kinase inhibitory activity causing an indirect effect on B cells, in such a way that it decreases IL-4 driven class switch recombination into IgG1, causing a reduction in IgG1 production (Siemasko et al., 1998; Claussen et al., 2012). Our results, with the increase in IgM and subtle decrease in IgG1 levels, are in accordance with the inhibition of this class switch. Similarly, in a clinical study comparing the serum immunoglobulin levels during teriflunomide and ofatumumab (human anti CD-20 monoclonal antibody) treatment, the proportion of patients with IgG levels below the lower limit of the normal and the proportion of patients with IgM levels below the lower limit of the normal were found to be 22.9% and 6.6% in teriflunomide using patients, and 14.2% and 17.7% in ofatumumab using patients respectively. It is interesting to see

that the proportion of IgG decrease with teriflunomide use was higher than the proportion of IgM decrease below the normal lower limits. The proportion of decrease in Ig G level with teriflunomid is also higher than that of ofatumumab, which is a drug totally active on B lymphocytes (Wiendl et al)., 2020).

In our study, an absolute clinical improvement was observed with teriflunomide use but exact mechanisms underlying this improvement seem difficult to explain. The impact of teriflunomide on B cells, either directly on B cells, or indirectly through CD4+ T cells might lead to the clinical ameloriation of the disease. The decrease in the number of plasma cells, the reduction of the mean CD 138+ expression (CD138 mean fluorescent intensity) meaning reduced activity of long lived mature plasma cells, as well as lower numbers of the helper T cells which may have taken a toll on B cell affinity maturation and the subtle decrease in IgG1, possibly resulting from the drug's inhibitory action on tyrosine kinase, might have caused the therapeutic changes in the immunological events at neuromuscular junctions. (Bortnick and Allman, 2013; Khodadadi et al., 2019; Nutt et al., 2015, A, A6). IgG1 is the major immunoglobulin in the pathogenesis of AChR autoantibody-mediated MG (Lefvert et al., 1981; Rodgaard et al., 1987) and may exert its effects via complement activation, by blocking AChR signaling, or by inducing internalization of the receptor from the cell membrane (Ey et al., 1979). IgM to IgG1 isotype switch is induced by IL-4 and IL-21 cytokines (Moens and Tangye, 2014), and whether their levels are altered after teriflunomide treatment *in vivo* requires further study.

In this study, it is exceptionally interesting to see that how a drug shows selectivity in its actions on cell types that is to say affecting helper T cells (even especially IFN-g secreting helper T cells) more than cytotoxic T cells, affecting mostly more mature plasma cells out of all B cells and affecting some types of immunoglobulins more. These findings are most probably due to some regulations and interactions of the immune mechanisms in relation to the effects and the dose of the drug. Clausen et al in their review explained that most of kinase inhibitory actions of teriflunomide were observed *in vitro* in higher concentrations (at least one order of magnitude higher) than those used to block DHO-DH, They also claimed that it is difficult to understand whether these effects would be reliable *in vivo* (Claussen et al., 2012). Indeed, our study may also show the higher concentration effects of the teriflunomide due to its use in a high dose. Likewise the differences of our findings from those of Yilmaz et al. may be explained by the differences in the doses of the drug, which was about 200-250 mg/day for each mouse in the current study and 30 mg/day in the Yilmaz et al's study (Yilmaz et al., 2021).

This study has a wide range of investigations with T cells, B cells and cytokines in the spleen, lymph node and the thymus in a detailed way and with serum anti-AChR immunoglobulins in the murine AChR-induced EAMG model. Nevertheless, due to some practical limitations, the memory T and B cells, and more elaborate studies in the thymus could not be performed. Furthermore the prolonging of the treatment period would yield more pronounced results regarding the immunoglobulins. Finally, the stimulation of T cells was preferred to be performed with PMA/Ionomycin rather than more specific AChR peptide stimulation, due to its capability of giving stronger signals as a valuable method.

In conclusion, our study shows that teriflunomide has clinical benefits and prevented the progression of the disease in a murine model of MG through different possible mechanisms including suppression of immune responses by reducing the number of cytokine-producing T cells, by changing the functions of plasma cells and by leading selective changes in anti-AChR antibody quantities and types. These mechanisms need to be verified with some other studies. Additionally, extending the follow-up period in future studies and performing the experiments with different doses of teriflunomide could definitely be more informative. The data presented herein suggests that teriflunomide may be a suitable candidate for use in MG patients and even in chronic inflammatory neuromuscular diseases owing to its widespread effect on the immune system and its low side effects. Further studies including human trials in MG patients would be beneficial.

Declarations

Authors' contributions

Emel Koseoglu, Ahmet Eken, Sabahattin Muhtaroglu conceived the study. Sabahattin Muhtaroglu provided funding. Emel Koseoglu carried out all the experiments. Emel Koseoglu and Ahmet Eken performed first immunizations. Emel Koseoglu, Ahmet Eken and Neslihan Sungur sacrificed the mice, harvested the organs. Ahmet Eken supervised all the experimental procedures post-sacrification and flow cytometry operation. Neslihan Sungur helped clinical scoring. Data generation and analyses were performed by Emel Koseoglu and Ahmet Eken. Flow cytometric data analyses on FlowJo and generation of flow plots were performed by Ahmet Eken. All the figures of the manuscript were generated by Emel Koseoglu, except Fig 1, Fig 4a, Fig 6a, Fig 8, which were generated by Ahmet Eken. Interpretation of data was performed by Emel Koseoglu and Ahmet Eken together. The manuscript was written by Ahmet Eken and Emel Koseoglu. All the authors read and contributed to the manuscript preparation, critical reading and approved the submitted version of the manuscript.

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Declaration of interest

The authors do not have any financial and personal conflict of interest.

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Figures

Fig 1

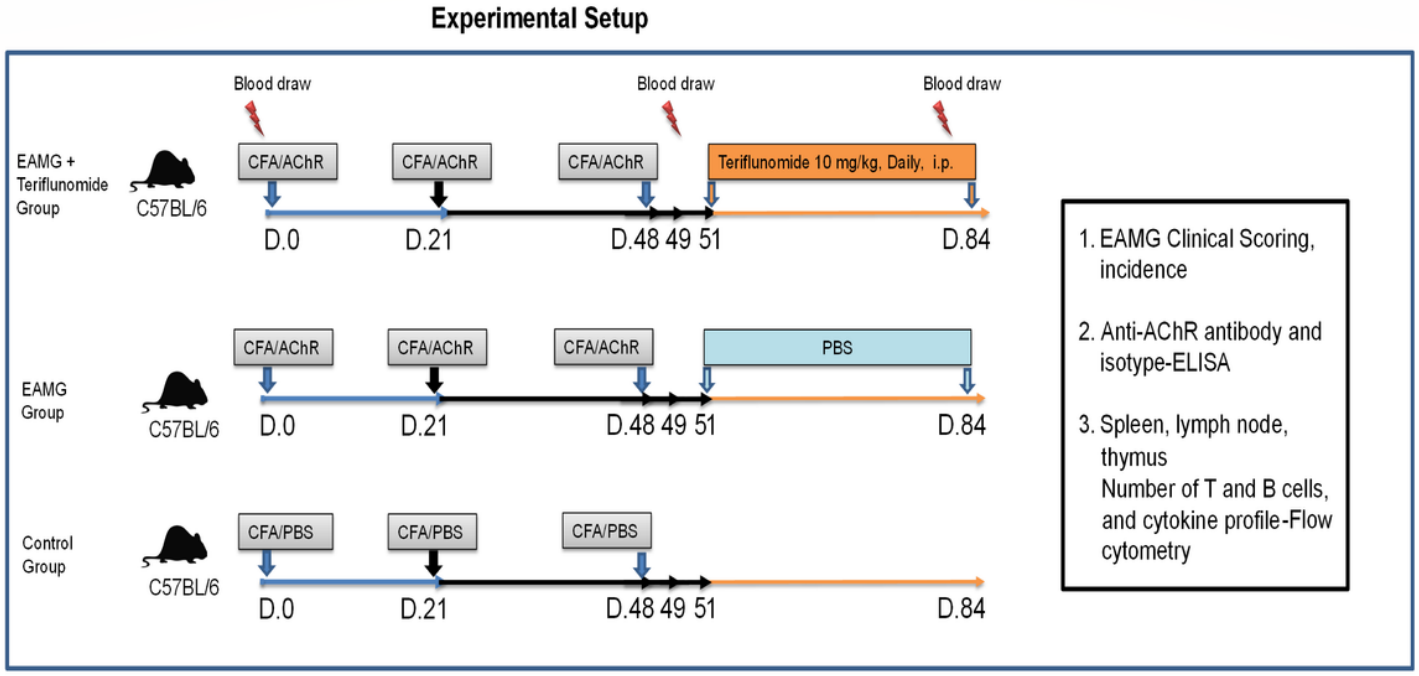

Figure 1

Experimental setup

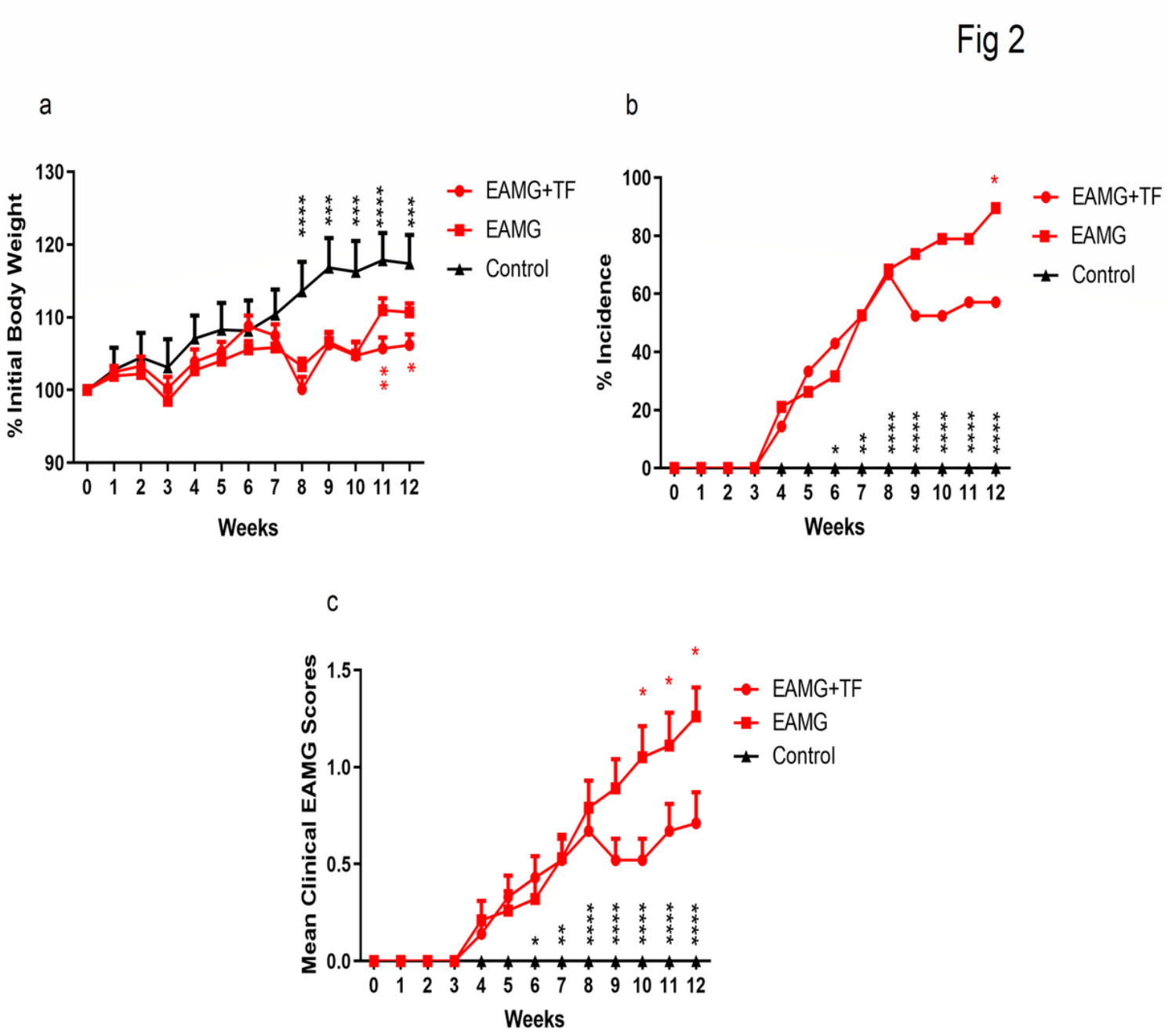

Figure 2

Teriflunomide ameliorates EAMG disease incidence and clinical disease scores. a) Weight loss, b) Incidence and c) Clinical disease scores in mice groups by week post-immunization, Error Bars indicate SEM, Control group n=10, EAMG+TF group n=21, EAMG group n=19. *: (*) Black asterisk indicates comparison of healthy control group to EAMG groups, whereas (*) Red asterisk indicates the comparison between EAMG+TF and EAMG groups. P-value<0.05, (*); p-value<0.005, (***); p-value<0.001, (****).

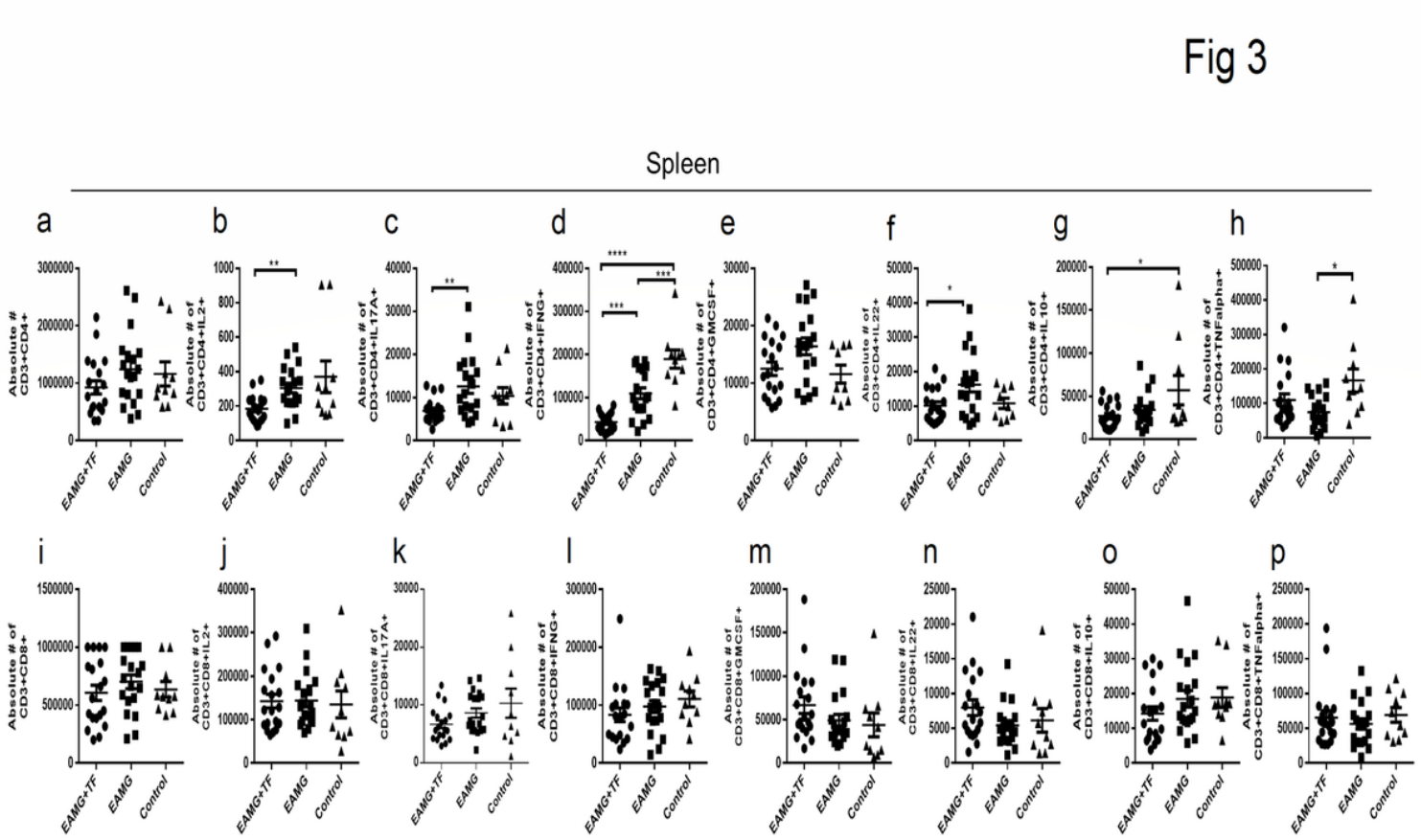

Figure 3

Teriflunomide inhibits splenic absolute numbers of IL-2+, IL-17A+, IFNG+ and IL-22 positive helper CD4+T cells. The absolute number of total CD4+ T cells and cytokine-producing subsets in the spleen of mice groups at the twelfth week post-immunization (a-h). Splenic CD8+ T cell numbers or their cytokine-producing subsets (i-p). Error bars indicate SEM, Control group n=10, EAMG+TF group n=21, EAMG group n=19. P-value<0.05, (*); p-value<0.01, (**); p-value<0.005, (***); p-value<0.001, (****).

Figure 4

a) Splenic gate strategy for a mouse from the control group is shown. b) The percent flow plots of total CD4+ T cells and cytokine-producing subsets in the spleen of mice groups at the twelfth week post-immunization. c) Mean fluorescent intensity (MFI) of IFN-γ produced by splenic CD4+ T cells. Error Bars indicate SEM, Control group n=10, EAMG+TF group n=21, EAMG group n=19. P-value<0.05, (*); p-value<0.001, (****).

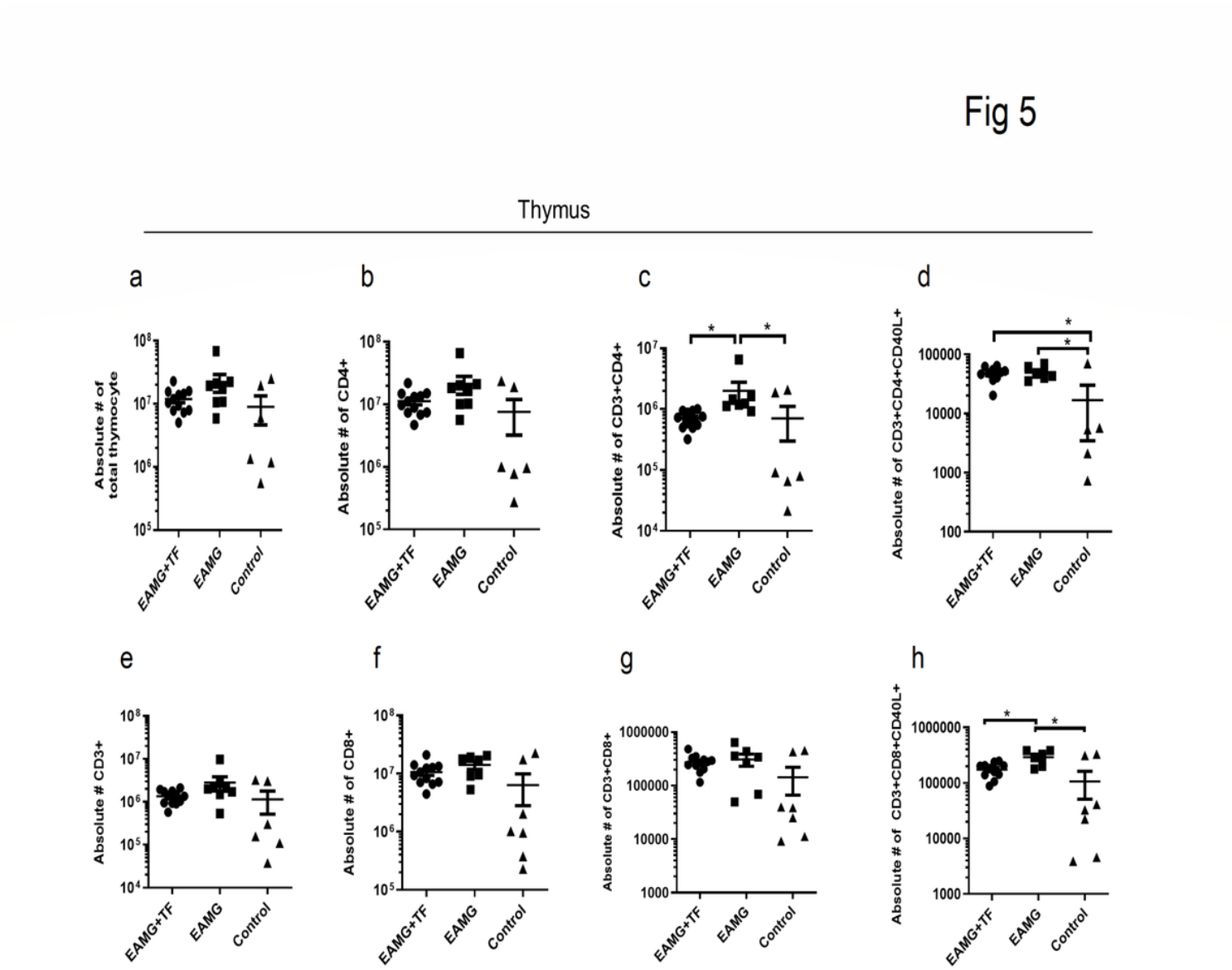

Figure 5

Teriflunomide inhibits lymph node absolute numbers of IFNG+, IL-10 and IL-22 positive helper T cells. The absolute number of total CD4+ T cells and cytokine-producing subsets in the lymph nodes (inguinal) of mice groups at twelfth week post-immunization (a-h). Lymph node CD8+ T cell numbers or their cytokine-producing subsets (i-p). Error bars indicate SEM, Control group n=10, EAMG+TF group n=21, EAMG group n=19. P-value<0.05, (*); p-value<0.01, (**); p-value<0.005, (***); p-value<0.001, (****).

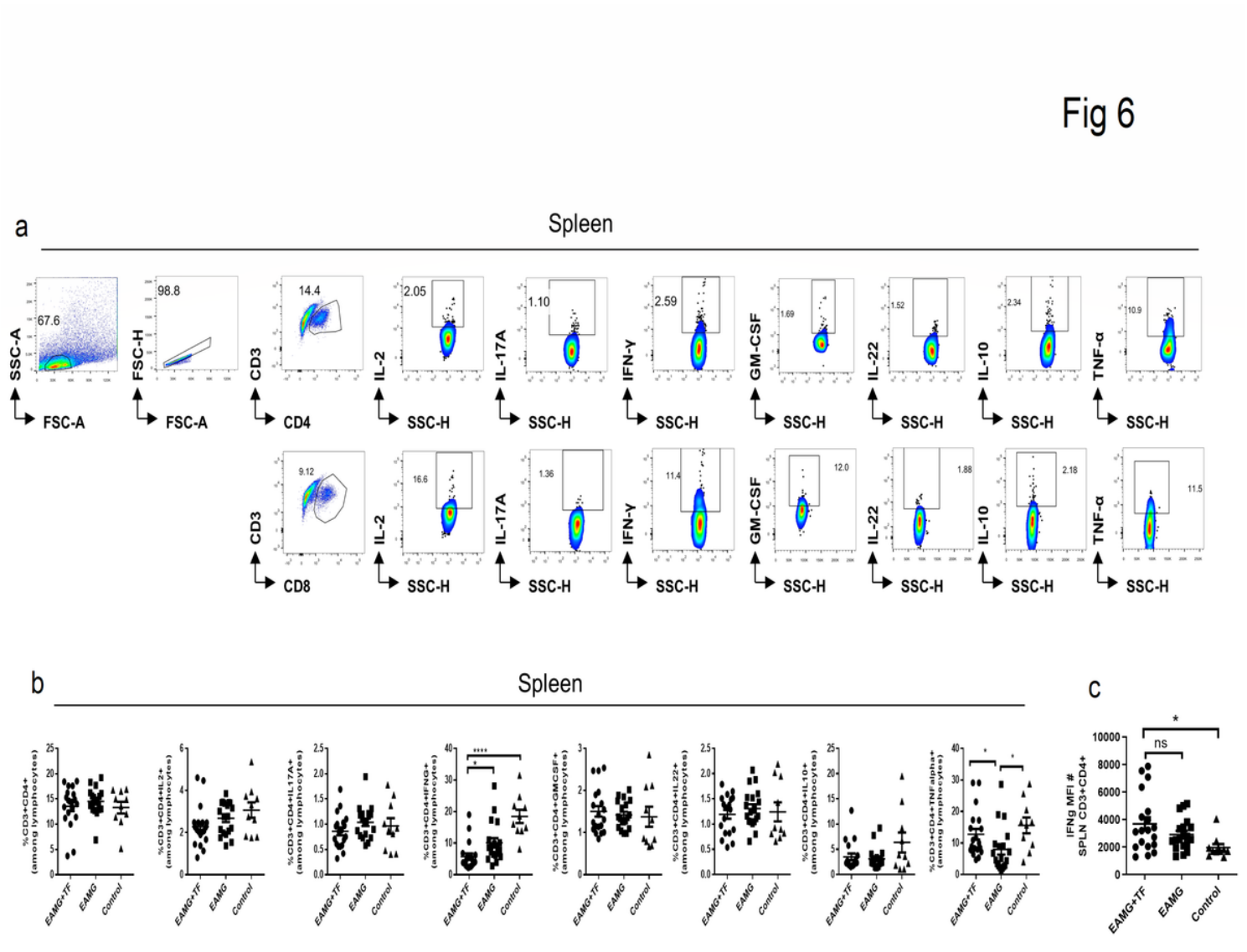

Figure 6

a) Lymph node gate strategy for a mouse from control group is shown. b) The percent flow plots of total CD4+ T cells and cytokine producing subsets in the lymph node of mice groups at the twelfth week post-immunization. c) Mean fluorescent intensity (MFI) of IFN-γ produced by lymph node CD4+ T cells. Error Bars indicate SEM, Control group n=10, EAMG+TF group n=21, EAMG group n=19. P-value<0.05, (*); p-value<0.01, (**); p-value<0.005, (***); p-value<0.001, (****).

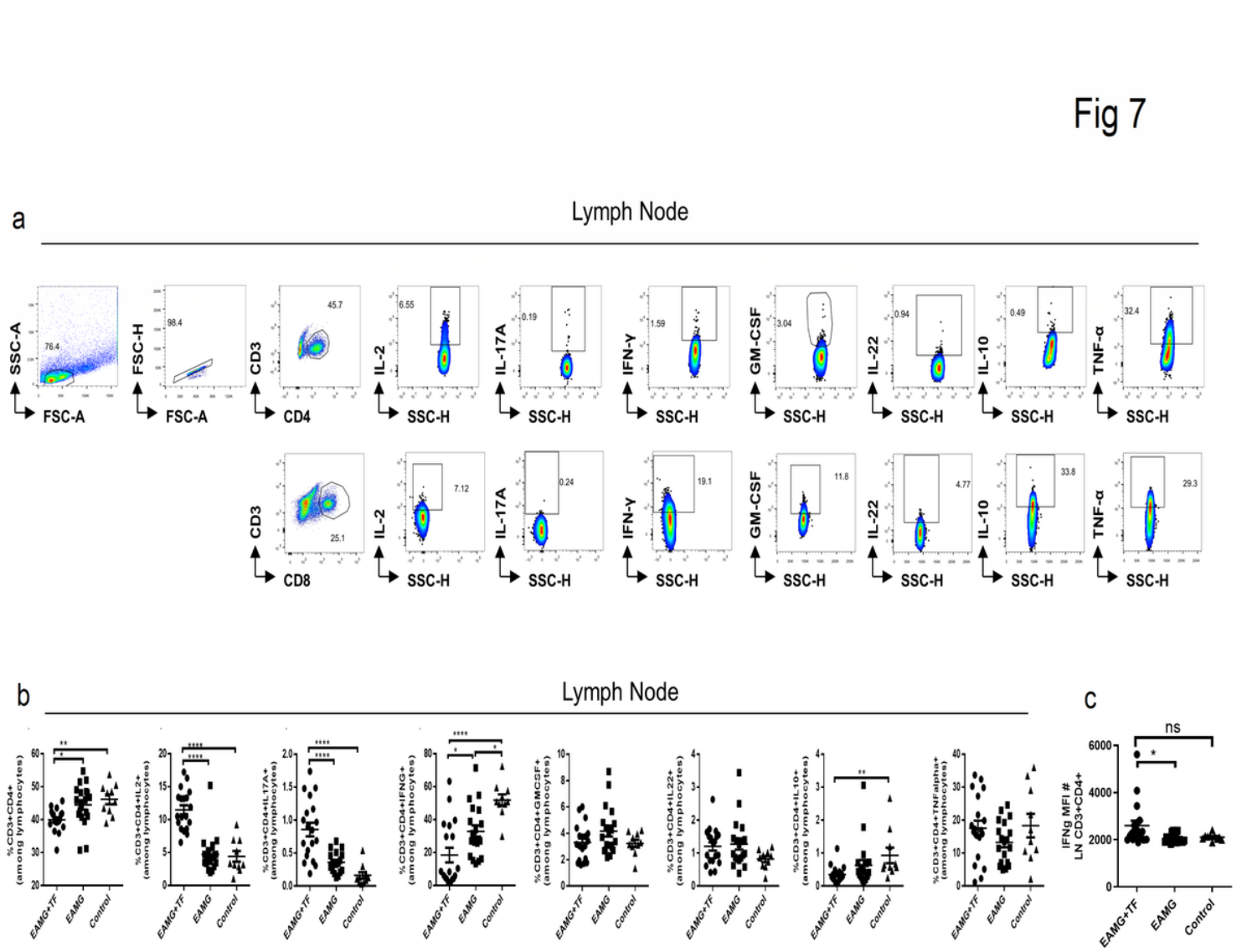

Figure 7

Teriflunomide reduces the absolute number of CD4+ but not CD8+ T cells in the thymus. Error bars indicate SEM, Control group n=7, EAMG+TF group n=10, EAMG group n=7. p-value<0.05, (*).

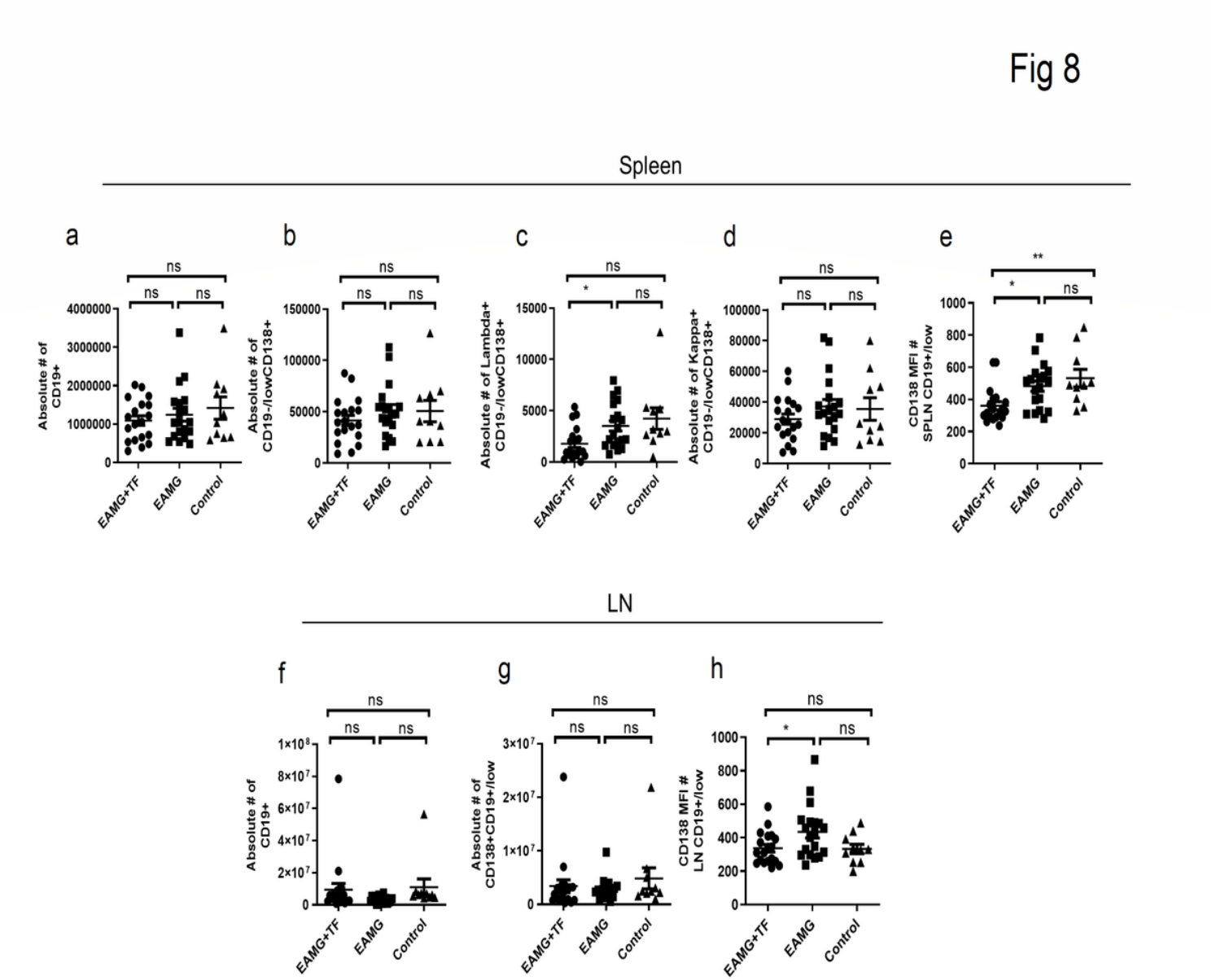

Figure 8

B cell gating strategies a) for spleen and b) lymph node are shown for a mouse from the control group.

Fig 9

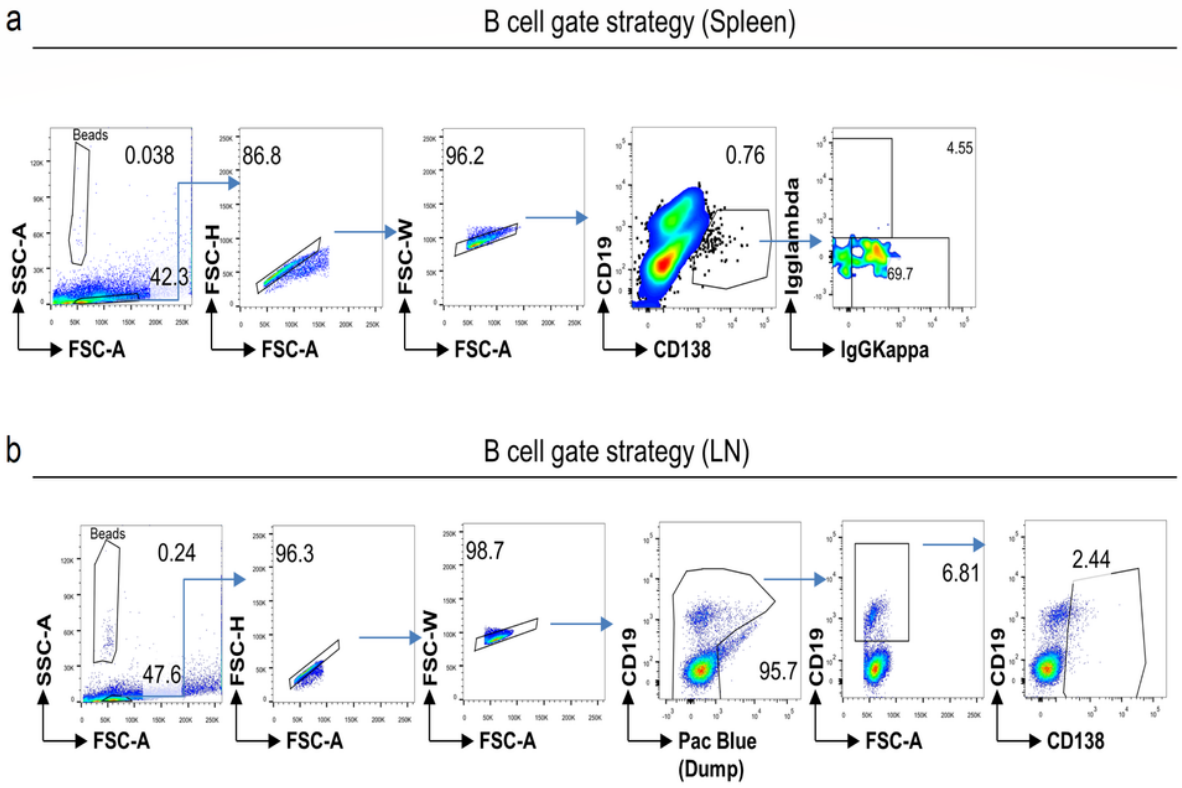

Figure 9

Teriflunomide reduces the absolute number of CD138+lambda+plasma cells in the spleen (a-e) but not in the lymph nodes (f-h). MFI of CD138 by $CD19^{+/low}$ cells is significantly lower in the EAMG+TF group in comparison to EAMG group (e and h). Error bars indicate SEM, Control group n=10, EAMG+TF group n=21, EAMG group n=19. P-value<0.05, (*); p-value<0.01, (**).

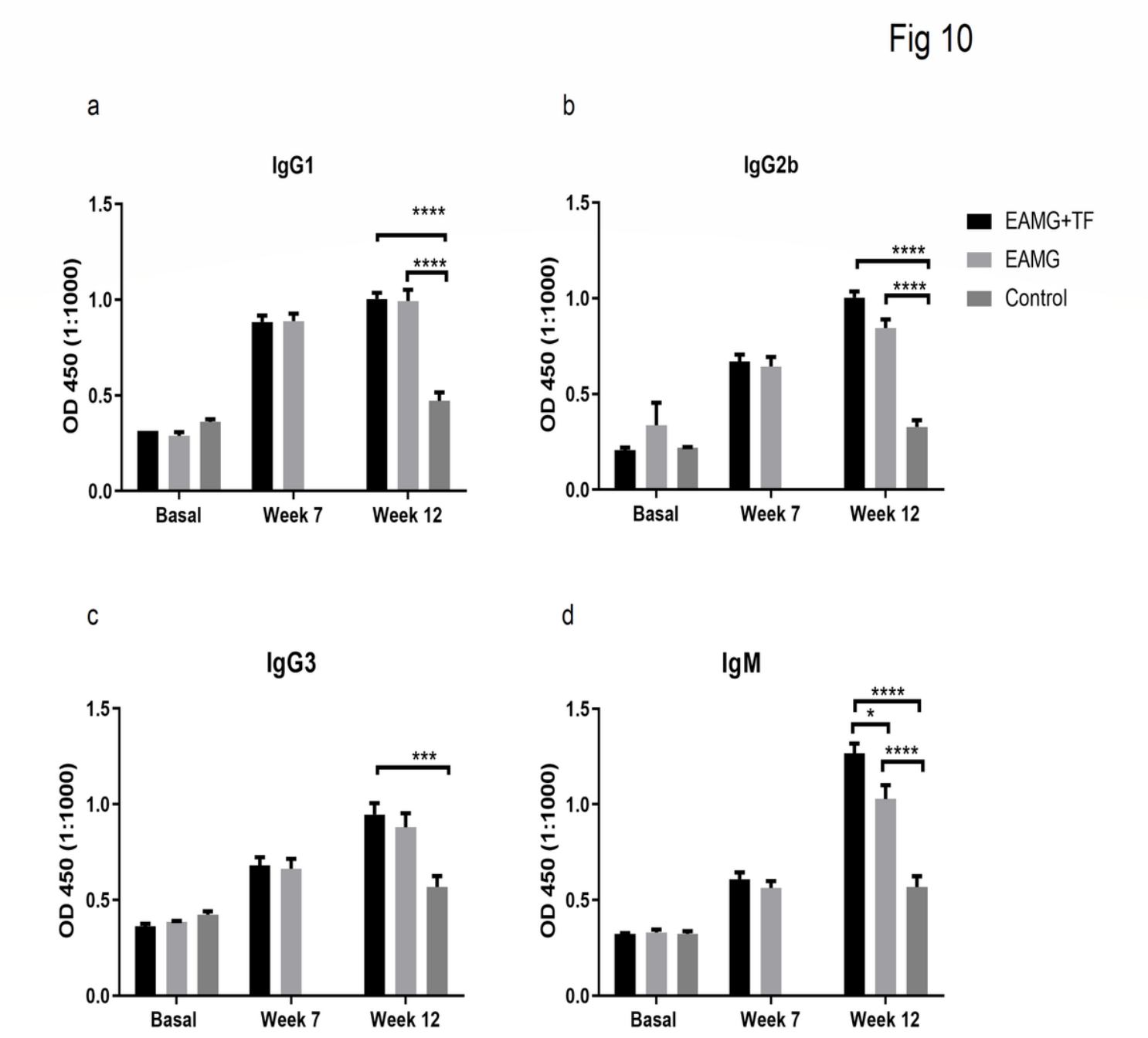

Figure 10

Teriflunomide increases anti-AChR IgM antibodies. IgG1, IgG2b, IgG3 and IgM serum levels measured by ELISA. Error bars indicate SEM, Control group n=10, EAMG+TF group n=21, EAMG group n=19. P-value<0.05, (*); p-value<0.005, (***); p-value<0.001, (****).

Fig 11

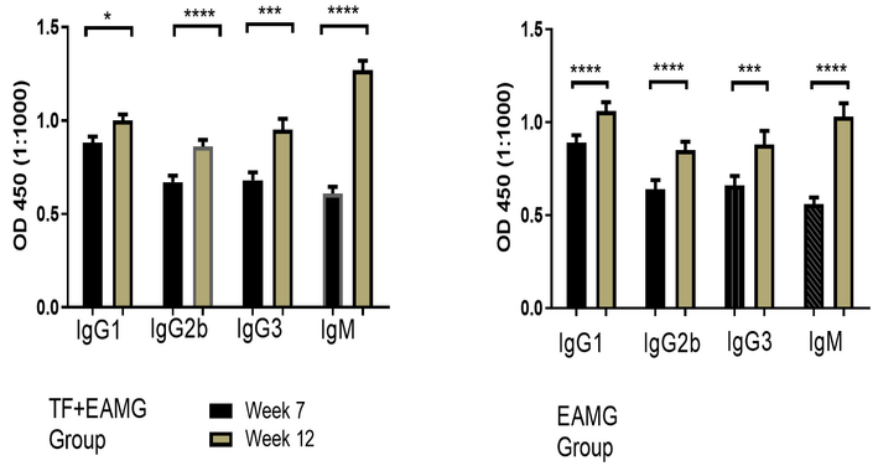

Figure 11

Comparison of IgG1, IgG2b, IgG3 and IgM levels within the disease model groups. Paired t-test was applied for each animal within the groups on the measurements at the 7 th and 12 th weeks. Error bars indicate SEM, Control group n=10, EAMG+TF group n=21, EAMG group n=19. P-value<0.05, (*); p-value<0.005, (***); p-value<0.001, (****).