Original Research Paper

Effect of switching glatiramer acetate formulation from 20 mg daily to 40 mg three times weekly on immune function in multiple sclerosis

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Abstract

Background: Many RRMS patients who had been treated for over 20 years with GA 20 mg/ml daily (GA20) switched to 40 mg/ml three times-a-week (GA40) to reduce injection-related adverse events. Although GA40 is as effective as GA20 in reducing annualized relapse rate and MRI activity, it remains unknown how switching to GA40 from GA20 affects the development of pathogenic and regulatory immune cells.

Objective: To investigate the difference in immunological parameters in response to GA20 and GA40 treatments.

Methods: We analyzed five pro-inflammatory cytokines (IL-1β, IL-23, IL-12, IL-18, TNF-α), and three anti-inflammatory/regulatory cytokines (IL-10, IL-13, and IL-27) in serum. In addition, we analyzed six cytokines (IFN-γ, IL-17A, GM-CSF, IL-10, IL-6, and IL-27) in cultured PBMC supernatants. The development of Th1, Th17, Foxp3 Tregs, M1-like, and M2-like macrophages were examined by flow cytometry. Samples were analyzed before and 12 months post switching to GA40 or GA20.

Results: Pro- and anti-inflammatory cytokines were comparable between the GA40 and GA20 groups. Development of Th1, Th17, M1-like macrophages, M2-like macrophages, and Foxp3 Tregs was also comparable between the two groups.

Conclusions: The immunological parameters measured in RRMS patients treated with GA40 three times weekly are largely comparable to those given daily GA20 treatment.

Introduction

Glatiramer acetate (GA) is an immunomodulatory drug used for the treatment of relapsing-remitting Multiple Sclerosis (RRMS).[1,2] GA primarily acts by curtailing relapse rate and subsequent disability in RRMS patients.[3] GA exerts its anti-inflammatory effect by affecting antigen presenting cells (APCs) to promote the development of Th-2 and production of anti-inflammatory cytokines (IL-4, IL-13, IL-10), and consequently, suppressing the development of pathogenic T cells (Th1, Th17, ThGM-CSF).[4,5]

GA was initially prescribed at a daily dose of 20 mg/ml via a subcutaneous injection (GA20). GA20 causes a significant increase in regulatory immune cells and a reduction in Th1 and Th17 cells.[2] As a consequence, GA20 causes a reduction in the number of enhancing lesions and their volume as detected by magnetic resonance imaging (MRI).[6] In addition, GA20 therapy results in significantly reduced relapse rates.[3,6,7] However, injection-related adverse events (IRAEs) impede treatment adherence and tolerability.[3,8] Subsequently it was demonstrated that a higher dose of 40 mg/ml administered 3 times a week (GA40) is equally effective as GA20 in reducing annual relapse rate (ARR) and MRI activity with reduced incidence of IRAEs.[9–13]

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Indeed, the incidence of injection site reaction (ISRs) in RRMS patients treated with GA40 was reduced by 20-50% compared to those treated with GA20.[10,14] However, it is unknown whether switching from GA20 to GA40 treatment affects the immunological parameters involved in the response to GA. In this study, we studied the difference in pro-inflammatory and regulatory immune responses generated by GA20 and GA40 treatments regimens.

Materials and methods

Subjects

Enrolled subjects in this study were participants in TEVA Pharmaceuticals clinical trial entitled "Open Label Study to Evaluate the Safety of Copaxone® and to Monitor the Neurologic Course of Disease in Multiple Sclerosis Patients Treated With Copaxone". This study is registered at clinical-trials.gov (NCT00203021). Participants who were originally randomized to the placebo group in the 01-9001 and/or the 01-9001E studies received glatiramer acetate 20 milligrams (mg) subcutaneous (SC) injection daily at the start of this study. After 18 July 2014 participants were offered the opportunity to continue treatment with glatiramer acetate 20 mg daily or switch to glatiramer acetate 40 mg three times weekly (TIW).

Participants were required to have the following study-specific baseline characteristics: a diagnosis of RRMS as defined by Poser et al 1983, at least 2 clearly identified relapses and remissions in the 2-year period prior to study entry, ambulatory with a Kurtzke EDSS score of 0 to 5.0 inclusive, and a stable neurologic state for at least 30 days prior to study entry. Patients provided written informed consent prior to undergoing study-related procedures. The study for blood collection was approved by study sites Institutional Review Boards and blood samples were transported overnight to our Laboratory in Piscataway, NJ. The immunological study using the collected blood was approved by Rutgers Institutional Review Board (Pro20140000929). The patient demographic information is shown in Table 1.

Analysis of cytokine production

Human PBMCs were isolated from whole blood by using lymphocyte separation medium (Thermo Fisher Scientific, Waltham, MA). The freshly isolated PBMCs were cultured at 4×10^6/ml with GA (Teva Pharmaceutical Industries Ltd., Israel) at $50 \mu g/ml$, LPS (InvivoGen, San Diego, CA) at $5 \mu g/ml$, or CD3/CD28 mAbs (Biolegend, San Diego, CA) at $2 \mu g/ml$ for 3 days, and cytokine production was examined by ELISA (Biolegend, San Diego, CA). The remaining cells were frozen and stored in liquid nitrogen. The serum was isolated from the blood by using BD Vacutainer (Becton Dickinson, Franklin Lakes, NJ), and the serum concentration of cytokines was examined by LEGENDplex™ (Biolegend, San Diego, CA).

Analysis of pathogenic and regulatory immune cells

The frozen PBMCs were thawed and cultured with CD3/CD28 mAbs in the presence of brefeldin A ($10 \mu g/mL$) and then stained with pooled CD4, IFN-γ, IL-17A, GM-CSF, and TNF-α mAbs, and percentages of CD4$^+$IFN-γ^+, CD4$^+$IL-17A$^+$, CD4$^+$GM-CSF$^+$, CD4$^+$TNF-α^+, were analyzed by flow cytometry (Supplementary Figure 1(a)). M1-like macrophages (CD14$^+$HLA-DR^{hi}) were examined by staining with CD14 and HLA-DR mAbs (Biolegend, San Diego, CA). M2-like macrophages (CD14$^+$CD206$^+$) were examined by staining with CD14 and CD206 mAbs (Biolegend, San Diego, CA). Naïve and memory Foxp3 Tregs were examined by staining with CD3, CD4, CD45RA, and Foxp3 mAbs (Biolegend, San Diego, CA).

Statistical analysis

The Shapiro-Wilk test was used to test the normality assumptions for the differences and the ratios of immunological parameters between baseline and 12 months post-baseline as well as the normality assumptions of the log transformed data. For the differences between baseline and 12 months post-baseline, Student's paired t-test or Signed Rank sum test were used depending on the normality assumptions holds or not. For the comparisons on the ratios of 12-month post-baseline and baseline between GA40 and GA20, since the normality assumptions do not hold, log-transformations were used first. If normality assumption held for the transformed data, either equal or unequal variance two-sample t-test was used depending on the test of homogeneity of variances between the two samples. If the normality assumption did not hold, Wilcoxon rank sum test was used for the comparison of transformed data between GA40 and GA20. All P-values ≤ 0.05 were considered statistically significant.

Results

Comparison of serum cytokine levels between GA20 and GA40

Blood samples were collected from RRMS patients before switching from GA20 to GA40 (baseline) and

Table 1. Patient demographic information.

Factor	GA20 (n = 16)	GA40 (n = 28)
Age (mean yr ± SD)	57±6	59±6
Women: Men (women %)	11:5 (69%)	18:10 (64%)
Duration of MS (mean yr ± SD)	27±3	32±5
Duration of GA medication (mean yr ± SD)	21±1	22±1
EDSS	2.8±1.6	3.4±2.5

12 months post switching (n = 28). Follow-up blood samples at 12 months were also obtained from RRMS patients who continued to take GA20 (n = 16). The demographics of the participants are shown in Table 1. We analyzed the pro-inflammatory cytokines; IL-1β, IL-23, IL-12, IL-18, IFN-γ, and IL-17A, and the anti-inflammatory/regulatory cytokines; IL-10, IL-13, and IL-27. A bead-based immunoassay was used to measure serum cytokine levels; and then, the change in the ratio for cytokine concentrations at 12 months of treatment relative to baseline was examined. We observed a decrease in this ratio for IL-1β, IL-23, IL-12, IL-18, IFN-γ, IL-17A, and TNF-α in RRMS patients treated with GA40 compared to those given GA20; however, the differences were not statistically significant except for serum TNF-α levels (Figure 1(b) and (d)). The change of ratio for TNF-α serum levels was significantly lower in GA40-treated patients compared to GA20-treated patients (Figure 1(d)).

Comparison of ex-vivo PBMC IL-10 production between GA20 and GA40

IL-10 production by PBMCs in response to GA is thought to contribute to the therapeutic mechanism of the drug.[15] Therefore, PBMCs isolated from MS patients treated with GA20 or GA40 were cultured with GA; and the production of IL-10, IL-17A, IFN-γ, and GM-CSF was measured by ELISA. We observed an increasing trend in IL-10 production 12 months post switching in both treatment groups (Figure 2(a)). However, the difference in the ratio for IL-10 production at 12 months relative to baseline between GA40 and GA20 groups was not significant (Figure 2(b)). We also examined the production of IL-10 by PBMCs in response to CD3/CD28 stimulation and LPS stimulation (Figures 3 and 4). Again, the change in ratio for IL-10 production in these conditions was not significantly different between the GA40 and GA20 groups (Figures 3(b) and 4(b)). Collectively, these data suggest that IL-10 production by PBMCs is comparable between GA20 and GA40 therapy.

Comparison of the development of pathogenic and regulatory immune cells

The development of Th1, Th17, and ThGM-CSF cells are associated with disease activity in RRMS. Therefore, we examined the development of Th1 (CD4+IFN-γ+ or CD4+TNF-α+), Th17 (CD4+IL-17+), and ThGM-CSF (CD4+GMCSF+) cells in RRMS patients treated with GA40 or GA20 at baseline and at 12 months post switching. The ratio of the percentage cell phenotype at 12 months relative to baseline was calculated for each subject. As shown in Figure 5, we did not observe a significant difference in the change of ratio for the development of Th1 (IFN-γ), Th1 (TNF-α), Th17, or ThGM-CSF cells between the two groups. Development of Foxp3 Tregs is associated with suppression of disease activity and progression. The naïve and memory Foxp3 Tregs possess suppressive activity[16] and GA-treatment can increase the development of naïve Foxp3 Tregs.[17] Therefore, we examined the development of naïve Foxp3 Tregs in RRMS patients given GA20 or GA40. As shown in Figure 6(a), we did not observe a significant difference in the development of naïve Foxp3 Tregs between GA20 and GA40 groups (Figure 6(a)). The development of memory Foxp3 Tregs was also not different between the two groups (Figure 6(a)). Next, we examined the development of M1-like (CD14+HLA-DRhi) and M2-like (CD14+CD206+) macrophages. M1-like macrophages play a pathogenic role in disease progression, while M2-like macrophages play a protective role in neuroinflammation.[18,19] As shown in Figure 6(b), there was no significant difference in the development of M1-like and M2-like macrophages between the GA20 and GA40 groups. These results suggest that development of pathogenic and regulatory immune cells are largely comparable between GA20 and GA40 treatments.

Discussion

GA is an immunomodulatory drug for patients with RRMS. Adverse events result from the high
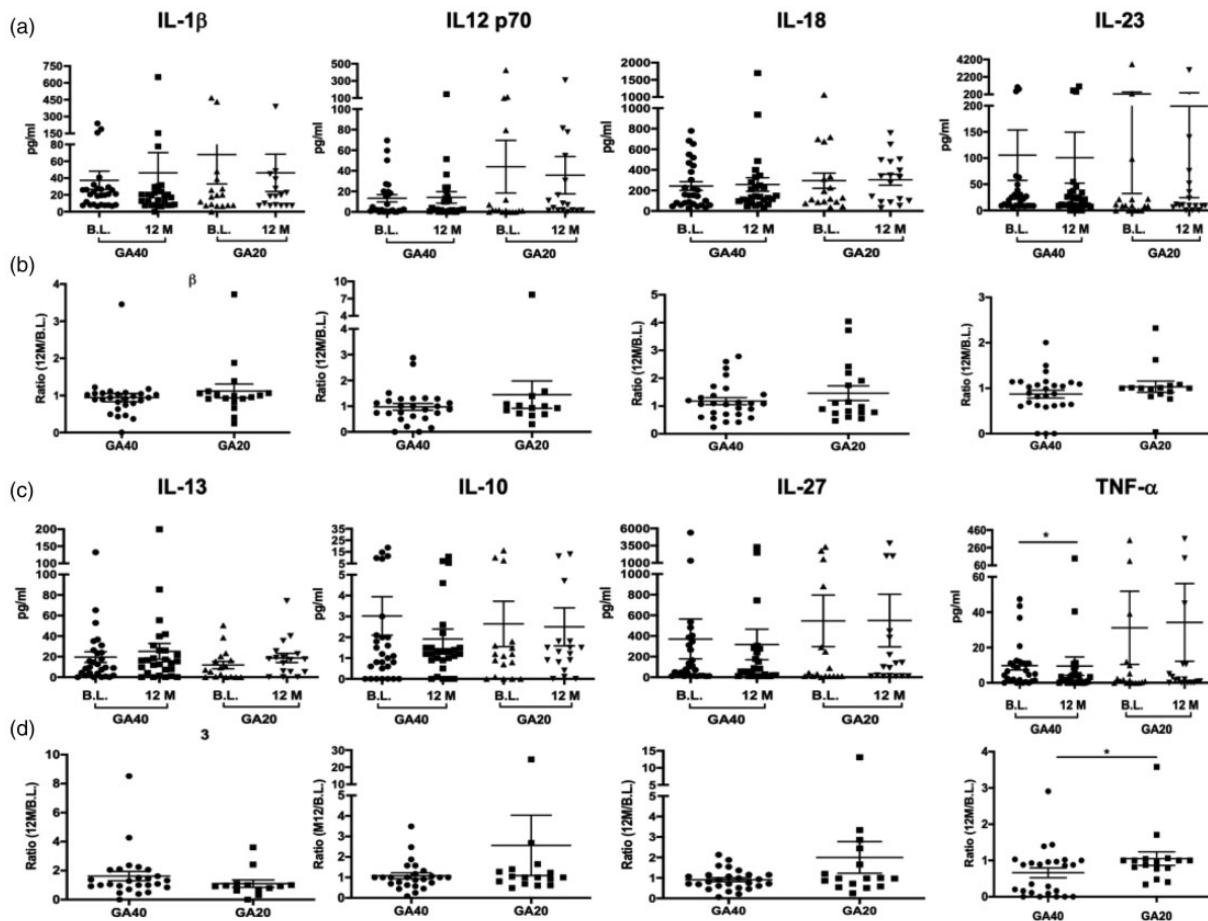
Figure 1. Cytokine concentration in serum. (a and c) Serum cytokine levels in RRMS patients treated with GA40 or GA20. Baseline (B.L.): before switching from GA20 to GA40. 12M: 12 months after switching to GA40. GA40; n=28, GA20; n=17. (b and d) Change in serum cytokine levels between B.L. and 12M. The cytokine ratio represents cytokine level at 12 M divided by that at B.L. for each subject *P< 0.05.
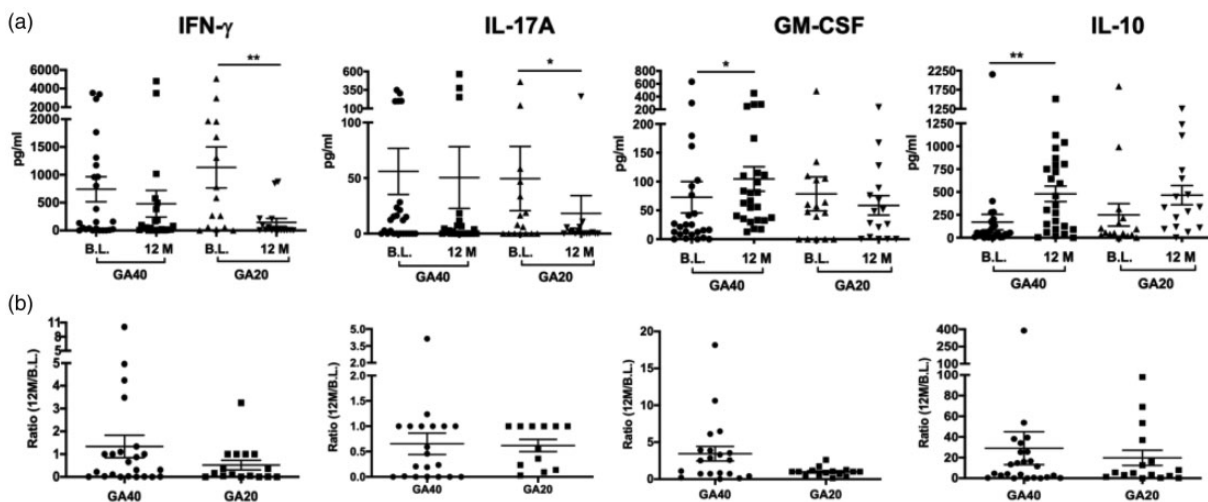

Figure 2. Cytokine production by PBMCs in response to GA. (a) PBMCs were cultured with GA at 50 μg/ml for 3 days, and production of cytokines was measured by ELISA. (b) Change in cytokine production between B.L. and 12M. Cytokine production at 12M is divided by that at B.L. *P< 0.05 **P< 0.01.
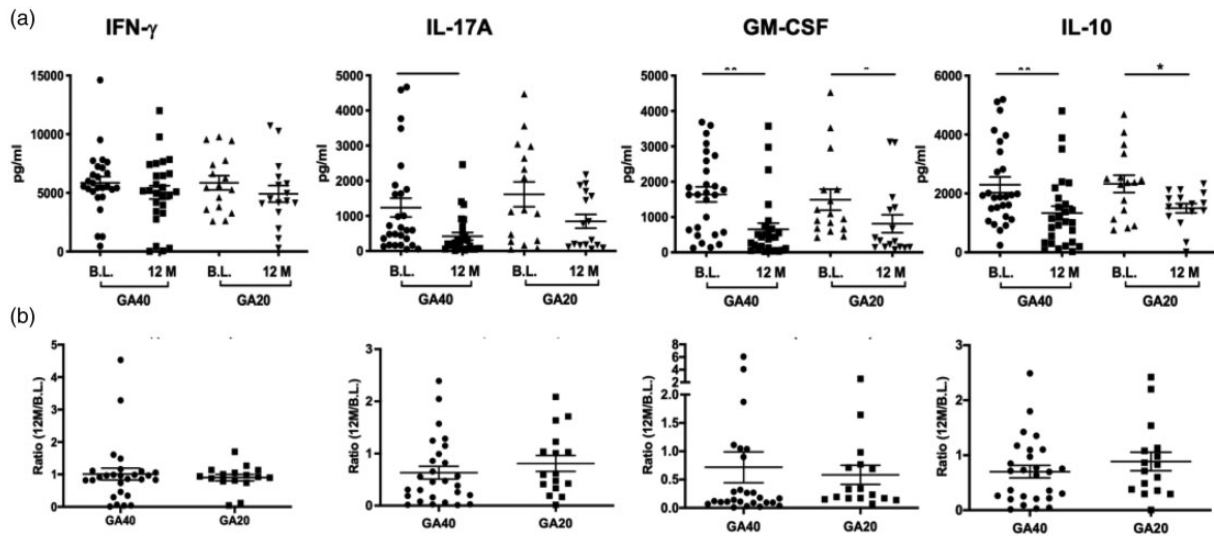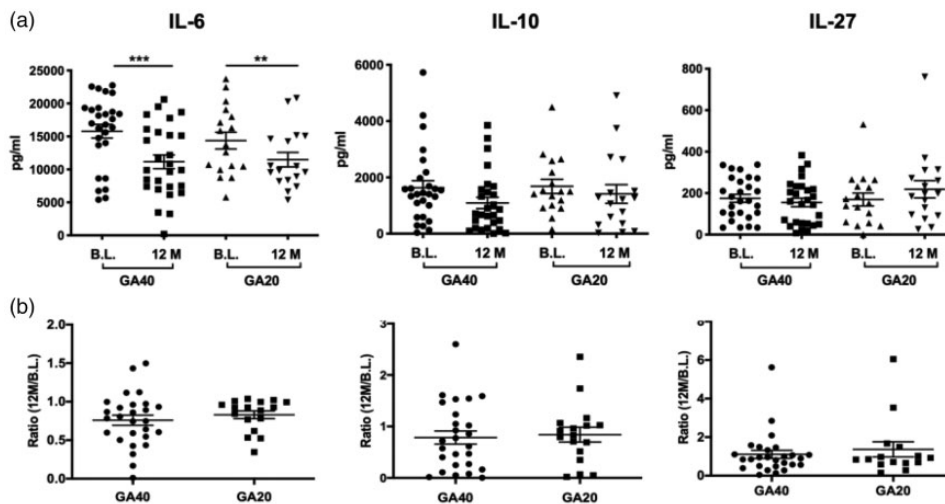
Figure 3. Cytokine production by PBMCs in response to CD3/CD28 mAbs. (a) PBMCs were cultured with CD3/CD28 mAbs at 2μg/ml for 3 days, and production of cytokines was measured by ELISA. (b) Change in cytokine production between B.L. and 12M. Cytokine production at 12M is divided by that at B.L. *P< 0.05 **P< 0.01.

Figure 4. Cytokine production by PBMCs in response to LPS. (a) PBMCs were cultured with LPS at 5μg/ml for 3 days, and production of cytokines was measured by ELISA. (b) Change in cytokine production between B.L. and 12M. Cytokine production at 12M is divided by that at B.L. **P< 0.01 ***P<0.001.

frequency of subcutaneous injections associated with a daily dose of GA at 20 mg/ml; hence, a higher dose of 40 mg/ml of GA administered 3 times a week was examined for therapeutic efficacy, lessening adverse events related to injection frequency, and potentially improved compliance. The reduced frequency of subcutaneous injections of GA40 led to a reduction of adverse events, but with sustained efficacy on clinical relapses and MRI activity.[14] One concern shared by physicians and patients alike is whether the reduced GA-injection frequency from daily to three time weekly in the long-run affects the

immune responses associated with the therapeutic mechanism of the drug; a question we intended to address in this study.

Serum cytokine levels are one of the immunological parameters associated with disease onset, relapse, and response to treatment in MS. The serum levels of IL-1β, IL-23, IL-12, IL-18, IL-13, IFN-γ, IL-17A, IL-27, and TNF-α are elevated in RRMS patients compared to healthy donors, while serum IL-10 levels are lower in RRMS.[20] Importantly, serum levels of IL-23, IL-12, IL-18, IL-13, IFN-γ,
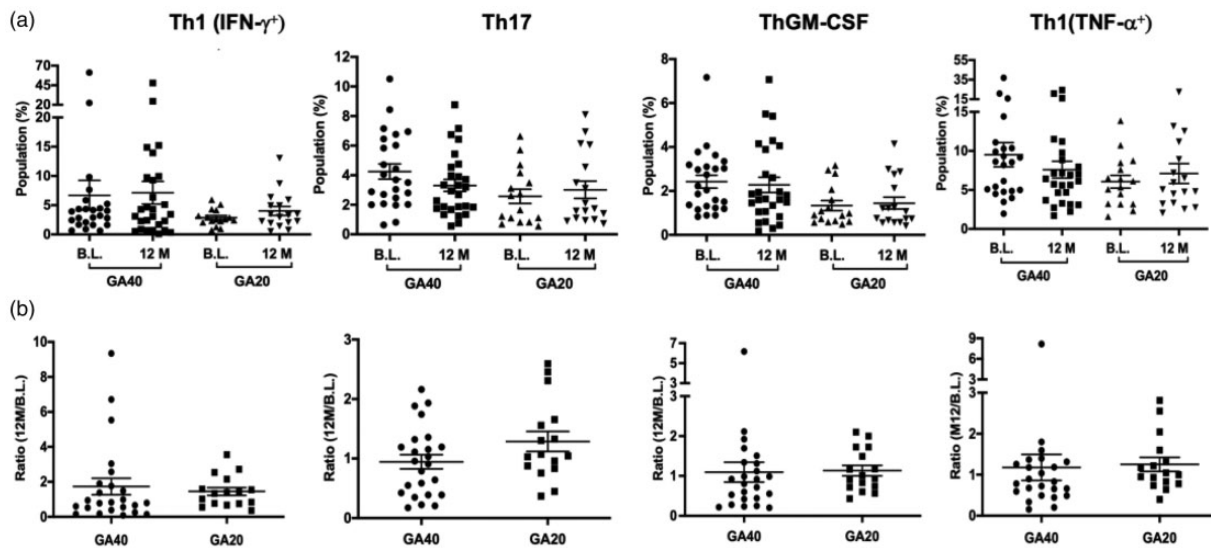
Figure 5. Development of Th1, Th17, and ThGMCSF cells in PBMCs. (a) PBMCs were cultured with CD3/CD28 mAbs in the presence of brefeldin A, and then stained with CD4, IFN-γ, IL-17A, GM-CSF, and TNF-α mAbs. The percentages of CD4$^+$IFN-γ$^+$, CD4$^+$IL-17A$^+$, CD4$^+$GM-CSF$^+$, CD4$^+$TNF-α$^+$ were analyzed by flow cytometry. (b) Change in the development of pathogenic T cells between B.L. and 12M. The population of pathogenic T cells at 12M is divided by that at B.L.
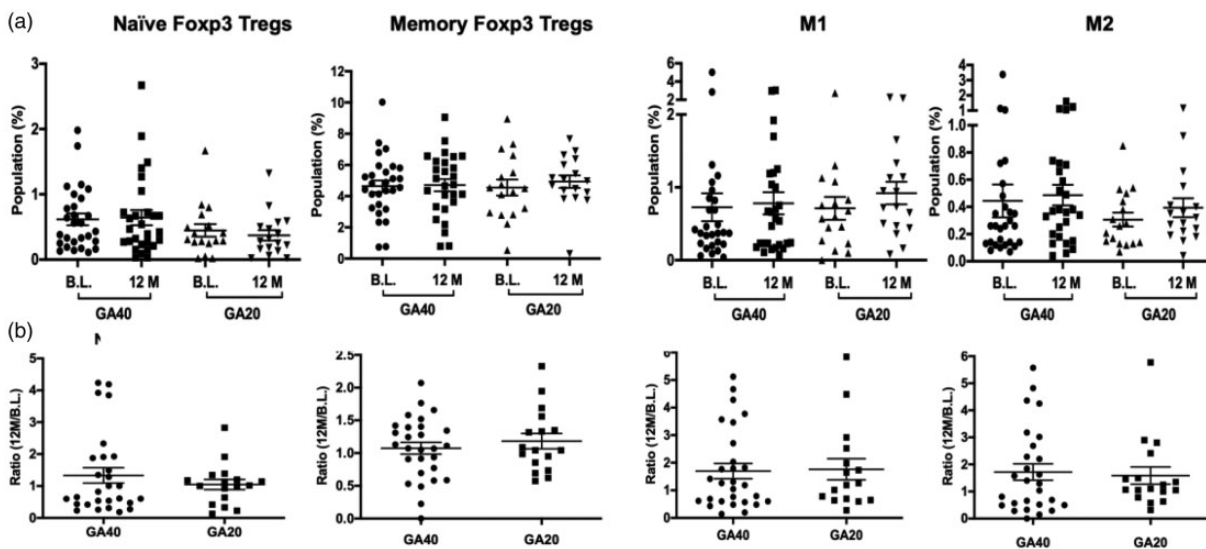

Figure 6. Development of M1/M2-like macrophages and Foxp3 Tregs in PBMCs. (a) PBMCs were stained with CD14, CD206, and HLA-DR mAbs. Percentages of CD14$^+$HLA-DR^{hi} (M1-like macrophages) and CD14$^+$CD206$^+$(M2-like macrophages) in PBMCs were analyzed by flow cytometry. (b) To analyze the development of Foxp3 Tregs, PBMCs were stained by CD3, CD4, CD45RA, and Foxp3 mAbs. Percentages of CD45RA$^+$CD4$^+$CD3$^+$Foxp3$^+$ (naïve Foxp3 Tregs) and CD45RA$^-$CD4$^+$CD3$^+$Foxp3$^+$ (memory Foxp3 Tregs) were analyzed by flow cytometry.

IL-17A, IL-27, and TNF-α are elevated upon relapse.[20–23] Interestingly, GA treatment reduces serum levels of IL-1β, IL-12, IL-18, IFN-γ, and IL-17A.[24–27] In contrast, serum levels of IL-10, IL-13, IL-27, and TNF-α are elevated with GA treatment.[26,28,29] Compared to those under GA20 therapy, a downward trend was observed for the serum levels of tested cytokines, except IL-13, in RRMS

patients treated with GA40 (Figure 1(b) and (d)). This indicates that a reduction in the frequency of GA administration is not associated with a reduction in the impact of GA40 on pro-inflammatory cytokines.

The induction of IL-10 is one of the presumed therapeutic mechanisms of action for GA. IL-10 is

produced by T cells, B cells, and myeloid cells; and GA treatment can promote the production of IL-10 from these leukocytes.[30] Therefore, we examined the production of IL-10 from PBMCs isolated from RRMS patients under GA40 and GA20 treatments by cultivation with GA (Figure 2), CD3/CD28 mAb (Figure 3), and LPS (Figure 4). We did not observe a significant difference in the change in ratio for IL-10 production at12 months relative to baseline between GA40 and GA20 treatments. Since IL-27 can induce the production of IL-10, and is a biomarker for clinical response to GA,[29] production of IL-27 in response to LPS was also examined. The change in ratio for IL-27 production was also not different between the GA40 and GA20 groups (Figure 4). These data suggest that the effect on IL-10 and IL-27-mediated immunomodulation is comparable between the GA40 and GA20 groups.

GA treatment can induce the development of type 2 antigen presenting cells (APCs) that promote the development of Th2 cells, and consequently, the production of anti-inflammatory cytokines. M2-like macrophages are one of the type 2 APCs that play a protective role in neuroinflammation.[18,19] In contrast, M1-like macrophages are pathogenic myeloid cells highly involved in neuroinflammation. GA treatment can promote M2-like macrophages and suppress the development of M1-like macrophages.[27] Therefore, we examined the development of M1-like and M2-like macrophages. The change of ratio (12 months relative to baseline) for both M1-like and M2-like macrophages was comparable between GA40 and GA20 groups (Figure 6(b)). Type 2 APCs can also promote the development of Foxp3 Tregs that play a pivotal role in suppression of disease onset and relapse. While the frequency of naïve and memory Foxp3 Tregs is low in RRMS patients compared to healthy donors,[31] GA treatment can promote the development of naive types of Foxp3 Tregs.[17,31] As shown in Figure 6(a), the population of memory Foxp3 Tregs is higher than that of naïve Foxp3 Tregs, and the change of ratio for naïve and memory Foxp3 Tregs was comparable between GA40 and GA20 groups. We also examined the development of Th1, Th17, and ThGM-CSF cells. Similarly, we did not observe a difference in change of ratio for these T cells between the two treatment groups (Figure 5).

Collectively, our results indicate that the immunologic effects of GA40 and GA20 treatment regimens are largely comparable as expected and that less frequent GA injections are not inferior to daily dosing

in generating the desired immunologic effects. These results are also consistent with the mechanism of action of GA and its clinical efficacy studies.

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