Signaling via toll-like receptor 4 and CD40 in B cells plays a regulatory role in the pathogenesis of multiple sclerosis through interleukin-10 production

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ABSTRACT

Background: B cells play an important role in the development of multiple sclerosis (MS), but can also exhibit regulatory functions through IL-10 production. Toll-like receptors (TLR) and CD40 signaling are likely to be involved in this process.

Objective: To investigate the ability of MS B cells to produce IL-10 in response to TLR stimulation in the presence or absence of CD40 co-stimulation.

Methods: Peripheral blood mononuclear cells obtained from 34 MS patients and 24 matched healthy participants (HS) were stimulated through either TLR4 or TLR9 alone, or together with CD40. Intracellular cytokine production was analyzed by flow cytometry.

Results: The frequency of IL-10-producing cells in total B cells after either TLR9 or CD40 stimulation was significantly lower in MS than HS, regardless of disease phase. The frequency of IL-10 producing B cells after TLR4 stimulation did not differ significantly between HS and MS, regardless of disease phase. TLR4 and CD40 co-stimulation synergistically increased the frequency of IL-10-producing but not pro-inflammatory cytokine-producing B cells at MS relapse. This effect was observed in both CD27$^-$ naïve and CD27$^+$ memory B cells. The frequency of IL-10-producing B cells following CD40 stimulation was significantly higher in interferon-β responders than non-treated MS patients. Finally, we confirmed that the frequency of IL-10-producing B cells positively correlated with IL-10 production quantity by B cells using magnetic-isolated B cells.

Conclusions: Cross-talk between TLR4 and CD40 signaling plays a crucial role in regulating IL-10 production by B cells during MS relapses, which may promote recovery from relapse. CD40 signaling in B cells is involved in the response to interferon-β in MS. Collectively, TLR4 and CD40 signaling in B cells may provide a promising target for MS therapy.

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1. Introduction

Multiple sclerosis (MS) is a complex immune-mediated demyelinating disease of the central nervous system [1]. Although MS is considered primarily a T cell-mediated disease, successful B cell depletion with anti-CD20 monoclonal antibodies suggests that B cells play an indispensable role in the pathogenesis of MS [2–4]. Additionally, it is reported that B cells play a regulatory role in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, and drive recovery from disease through IL-10 production. Chimeric mice with IL-10 deficiency only in B cells display persistent pathogenic T cell responses and develop chronic EAE without clinical recovery [5], similar to observations of B cell-deficient mice [6]. IL-10-producing B cells have recently been identified in humans [7], and several studies indicate the decreased capacity of B cells to produce IL-10 in MS [8–10]. These findings indicate that triggering IL-10 production by B cells could be a novel therapeutic strategy to limit pathogenic T cell responses in MS.

Epidemiological studies indicate an association between infection and MS relapses [11]. Although the exact mechanism for this is not fully understood, recognition of pathogen-derived molecules by toll-like receptors (TLR) provides accessory signals for activation of adoptive immunity [12–14]. However, infection is not always harmful in MS. Some infections are shown to trigger the development of IL-10-producing B cells via TLR and thereby suppress autoimmunity. TLR4 agonists contained in the helminth parasite *Schistosoma mansoni* can induce IL-10 production by B cells [15], and B cells from helminth-infected patients produce higher levels of IL-10 compared with uninfected patients, which is associated with a lower risk for MS relapse [16,17]. Furthermore, TLR agonists contained in *Mycobacterium tuberculosis* are involved in EAE recovery through IL-10 production by B cells [18], and the *Mycobacterium bovis* strain Bacillus Calmette-Guerin reduces disease activity detected by Magnetic Resonance Imaging (MRI activity) and the risk for conversion from clinically isolated syndrome to clinically definite MS [19–21]. In addition to TLR signaling, CD40 signaling is crucial for the differentiation of IL-10-producing regulatory B cells [22], and mice with CD40 deficiency restricted to B cells fail to recover from EAE, similar to mice with IL-10-deficient B cells [5]. These observations indicate that TLR including TLR4 and CD40 signaling are essential pathways to trigger the development of IL-10-producing B cells despite previous demonstrations that impaired IL-10 production by MS B cells was limited through TLR9 and CD40 stimulation [8–10].

To elucidate the individual and combined effects of TLR and CD40 signaling in regulating IL-10 production by MS B cells, we analyzed IL-10-producing B cells in HS and MS after TLR stimulation in the presence or absence of CD40 co-stimulation.

2. Materials and methods

2.1. Subjects

Thirty-four patients with relapsing-remitting MS and twenty-four age- and sex-matched healthy participants (HS) were enrolled in the study (Table 1). All MS patients met the revised McDonald criteria [23] and were negative for anti-aquaporin-4 antibody. Seventeen MS patients were treated with interferon (IFN)-β; the others were not treated with disease-modifying drugs. In this study, "in remission" was defined as a clinically stable state of more than 30 days. "At relapse" was defined as the period within 14 days after the initiation of a neurological exacerbation; this "exacerbation" indicates neurological episodes lasting more than

24 h. Sampling was performed both in remission and at relapse in six MS patients. IFN-β responders were defined as having neither relapses nor MRI activities within 1 year before sampling, and the others were defined as non-responders.

The study was approved by the Medical Ethics Committee of Kyoto University of Medicine and was conducted in accordance with the principles contained within the Declaration of Helsinki. All participants provided informed consent.

2.2. Sample collection and cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood samples using a Ficoll gradient with lymphocyte separation medium 1077 (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. CD19-positive B cells were isolated from PBMC using magnetic cell separation (MACS; Miltenyi Biotec) according to the manufacturer's protocol. B-cell purity was higher than 93% as assessed by flow cytometry. PBMC or isolated B cells were cultured at a density of 2×10^5/well for 72 h in AIM-V (Thermo Fisher Scientific, Tokyo, Japan) in 96-well flat-bottom plates. Cells were stimulated with 1 μg/ml CD40 ligand (Miltenyi Biotec, Auburn, CA, USA), 10 μg/mL lipopolysaccharide (Enzo Life Sciences, Farmingdale, NY, USA), or 0.1 μM cytosine−phosphate−guanosine oligodeoxyribonucleotide 2006 (Miltenyi Biotec) as indicated. Phorbol 12-myristate 13-acetate/ionomycin cell stimulation cocktail (eBioscience, Hatfield, UK) and Brefeldin A solution (eBioscience) were added during the last 6 h of cell culture.

2.3. Flow cytometric analysis of intracellular cytokines in B cells

Stimulated PBMC were harvested, washed, and stained with anti-human CD19 phycoerythrin (PE)-cyanine7 (Cy7) (SJ25C1) (BD Biosciences, San Jose, CA, USA). For intracellular staining, cells were washed again, fixed, permeabilized, and stained with anti-human antibodies. Anti-human antibodies included anti-IL-10 PE (JES3-9D7), anti-tumor necrosis factor (TNF)-α peridinin-chlorophyll protein−cyanine5.5 (Mab11), anti-TNF-β (lymphotoxin α, LT) APC (359-81-11) (all from eBioscience), anti-IL-6 PE (AS12) (Becton Dickinson, Franklin Lakes, NJ, USA), and control isotype-matched antibodies. Data were acquired using a FACS Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA) to enable to frequency of cells in the whole population to be calculated.

2.4. Characterization of IL-10-producing B cells by flow cytometry

Phenotypic characteristics of IL-10-producing B cells were analyzed by flow cytometry in 10 HS and 20 MS patients (14 in remission and six at relapse). Stimulated PBMC were harvested, washed, and stained for cell surface molecules and intracellular IL-10 as previously described. Anti-human antibodies included anti-CD40 fluorescein isothiocyanate (5C3), anti-TLR4 APC (HTA125), anti-TLR9 PE (eB72-1665) (all from eBioscience), anti-CD19 PE-Cy7 (SJ25C1) (BD Biosciences), anti-CD27 APC-Cy7 (O323) (BioLegend, San Diego, CA, USA), and control isotype-matched antibodies.

2.5. Measurement of IL-10 secreted from PBMC or isolated B cells

Isolated B cells or whole PBMC were cultured at a density of 2×10^5/well for 72 h in AIM-V in 96-well flat-bottom plates with CD40 ligand and lipopolysaccharide. Subsequently, the supernatant was collected. IL-10 concentration therein was assayed using

Table 1
Demographic and clinical characteristics of healthy participants and multiple sclerosis patients.

Characteristic	Healthy Participants (n = 24)	Multiple Sclerosis	
		remission (n = 24)	relapse (n = 16)
Age (mean ± SD)	34 ± 7.1	39 ± 11	40 ± 10
Female/male	19/5	21/3	13/3
Disease duration (mean ± SD)	–	9.3 ± 8.3	6.3 ± 5.4
Number of relapses[a] (mean ± SD)	–	0.6 ± 0.8	0.5 ± 0.5
EDSS score (mean ± SD)	–	2.3 ± 1.5	2.1 ± 1.2
Disease modifying drug None	–	11	7
IFN-β	–	13	9

[a] Number of relapses within 1 year before sampling. EDSS = expanded disability status scale; IFN-β = interferon-β; SD = standard deviation.

Cytometric Beads Array Enhanced Sensitivity Flex Set System (BD Biosciences) according to the manufacturer's protocol.

2.6. Statistical analysis

Data are mean ± standard deviation (SD). Statistical analysis used nonparametric methods: the Kruskal–Wallis test for comparisons of more than two unpaired groups, the Mann–Whitney U test for comparisons of two unpaired groups, and the Wilcoxon paired rank test for analysis of paired data, and the Spearman rank test and the linear regression analysis for correlation analysis. All data sets were analyzed using GraphPad Prism software (GraphPad, San Diego, CA, USA). A p-value <0.05 was considered statistically significant.

3. Results

3.1. TLR- and CD40-mediated IL-10 production by B cells in MS patients

Both TLR4 and TLR9 engagement can stimulate B cells to produce IL-10 [7,24]. Thus, we first evaluated the ability of B cells from MS patients to produce IL-10 after TLR4 or TLR9 stimulation. The frequency of IL-10-producing cells in the total CD19$^+$ B cell population after TLR4 stimulation did not differ significantly between HS and MS, regardless of disease phase (Fig. 1A). However, it was significantly lower in MS than HS after TLR9 stimulation, regardless of disease phase (14% in HS, 9.9% in MS remission, $p = 0.006$; 7.7% at MS relapse, $p = 0.0025$). These results suggest that TLR4 and TLR9 have distinct roles in regulating IL-10 production by B cells in MS.

We also found that the frequency of IL-10-producing cells in total CD19$^+$ B cells after CD40 stimulation was significantly lower in MS than HS, regardless of disease phase (4.9% in HS, 1.9% in MS remission, $p = 0.018$; 1.8% at MS relapse, $p = 0.022$) (Fig. 1A).

3.2. Effect of co-stimulation through TLR and CD40 on IL-10 production by B cells in MS patients

We evaluated the synergistic effect of co-stimulation through TLR and CD40 on IL-10 production by B cells. Compared with the single stimulation of TLR4, the dual stimulation of TLR4 and CD40 significantly increased the frequency of IL-10-producing B cells in both HS and MS relapse but not in MS remission (from 1.0% to 4.6% in HS, $p = 0.0002$; from 0.74% to 1.6% in MS remission, $p = 0.22$; from 1.1% to 3.8% at MS relapse, $p = 0.026$) (Fig. 1A). Compared with single stimulation of CD40, dual stimulation increased the frequency of IL-10-producing B cells only at MS relapse from 1.8% to 3.8%, although this difference did not reach statistical significance. The frequency of IL-10-producing B cells after the dual stimulation was significantly lower in MS remission than HS and MS relapse ($p = 0.001$ and $p = 0.018$, respectively). Intra-individual analysis confirmed the frequency of IL-10-producing B cells after dual stimulation was significantly lower in MS remission than at relapse (6.2% at relapse and 2.1% in remission, $p = 0.031$) (Fig. 1C).

We also found that the dual stimulation of TLR9 and CD40 significantly increased the frequency of IL-10-producing B cells in all three groups compared with single stimulation of CD40 ($p < 0.0001$, respectively), but not of TLR9. Of note, the frequency of IL-10-producing B cells after dual stimulation was significantly lower in MS than HS, regardless of disease phase ($p = 0.0002$ in MS remission; $p = 0.0034$ at MS relapse). Intra-individual analysis of MS patients confirmed no significant difference in the frequency of IL-10-producing B cells between relapse and remission phases after the dual stimulation of TLR9 and CD40 (Fig. 1D).

3.3. Frequency of IL-10-producing cells in naïve and memory B cells in MS patients

After TLR4 stimulation, the frequency of IL-10-producing cells in both CD27$^-$ naïve and CD27$^+$ memory B cells did not differ significantly among the three groups (Fig. 2A and B). After CD40 stimulation, the frequency of IL-10-producing cells in CD27$^-$ naïve B cells was significantly lower in MS than HS, regardless of disease phase (2.1% in HS, 1.2% in MS remission, $p = 0.0055$; 0.52% at MS relapse, $p = 0.016$) (Fig. 2A), whereas the frequency in CD27$^+$ memory B cells did not differ significantly among the three groups (Fig. 2B). Dual stimulation of TLR4 and CD40 significantly increased the frequency of IL-10-producing cells in CD27$^-$ naïve B cells in all the three groups compared with single stimulation of either TLR4 or CD40 (Fig. 2A). This synergistic effect was even more pronounced at MS relapse than in HS (4.1% in HS and 6.8% at MS relapse), but was significantly lower in MS remission (1.8%) than HS and MS relapse ($p = 0.0039$ and $p = 0.029$, respectively). In contrast to CD27$^-$ naïve B cells, the dual stimulation of TLR4 and CD40 significantly increased the frequency of IL-10-producing cells within the population of CD27$^+$ memory B cells only at MS relapse compared with single stimulation of either TLR4 or CD40, and this was significantly higher than in HS (4.9% in HS and 14% at MS relapse, $p = 0.013$) (Fig. 2B). The dual stimulation also tended to increase the frequency of IL-10-producing cells in the CD27$^+$ memory B cell population in MS remission, but without statistical significance.
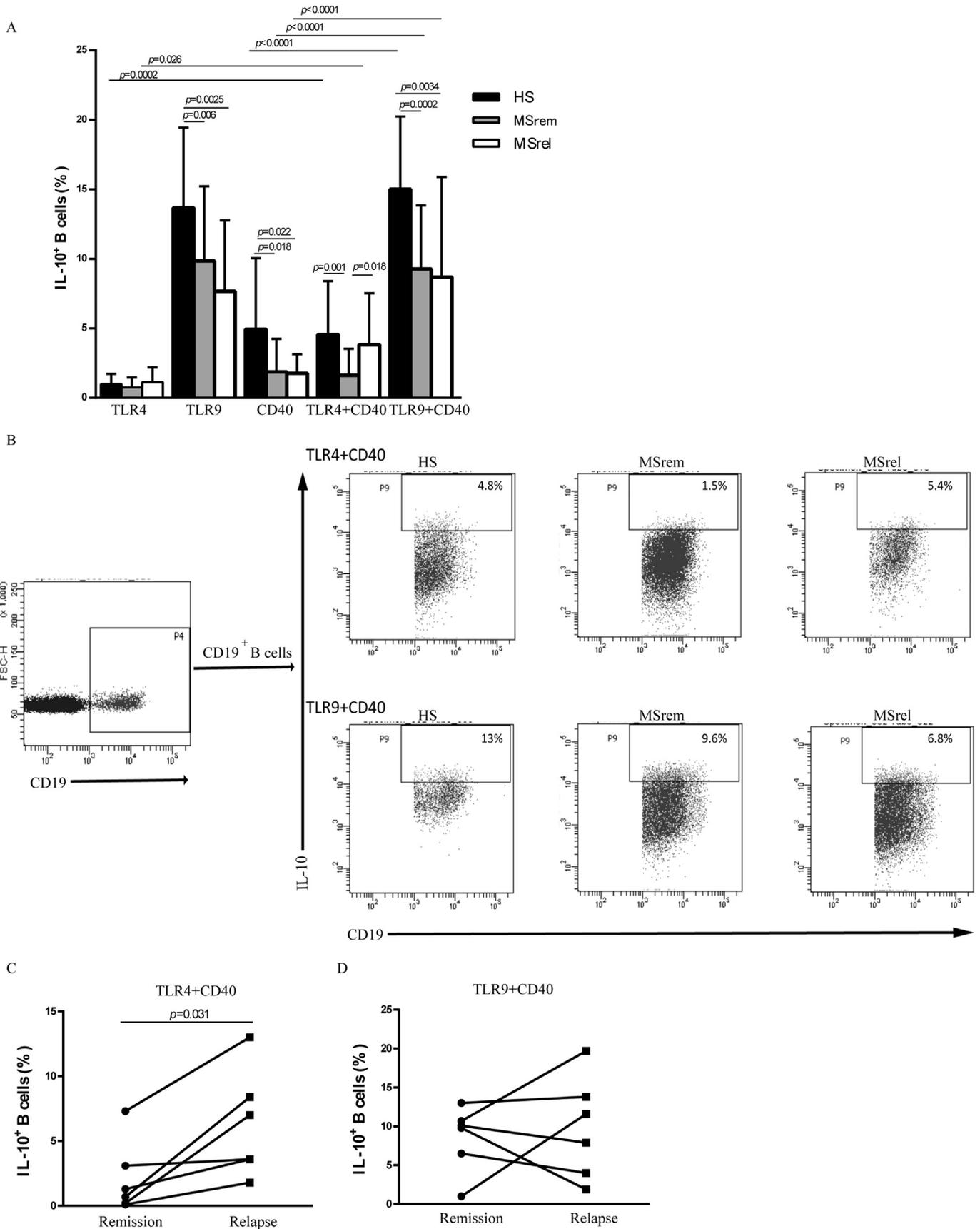
Fig. 1. Numbers of IL-10-producing cells in total CD19+ B cells after TLR stimulation in the presence or absence of CD40 co-stimulation. Peripheral blood mononuclear cells were stimulated through TLR and CD40 as indicated. Cells were stained for surface CD19 and intracellular IL-10. (A) Bar graphs show mean (±standard deviation) frequencies of IL-10-positive cells in CD19+ B cells determined by flow cytometry in the indicated groups. (B) Representative analysis showing the frequency of IL-10-producing cells in CD19+ B cells

3.4. B cell subset distribution and the frequency of IL-10-producing naïve and memory B cells in MS patients

The frequency of CD27$^+$ memory B cells within the CD19$^+$ B cell population was significantly lower in MS remission than HS cells at baseline (17% in HS and 7.8% in MS remission, $p = 0.0005$) (Fig. 2C). After TLR4 stimulation, the frequency of CD27$^+$ memory B cells did not change significantly in all the three groups compared with baseline. Although CD40 stimulation did not have a statistically significant effect on the frequency of CD27$^+$ memory B cells in all of the three groups compared with baseline, the frequency of CD27$^+$ memory B cells was significantly lower in the MS group compares to HS, regardless of disease phase (18% in HS, 5.7% in MS remission, $p = 0.0007$; 6.8% at MS relapse, $p = 0.015$). The dual stimulation of TLR4 and CD40 significantly increased the frequency of CD27$^+$ memory B cells only in HS compared with single stimulation of either TLR4 or CD40, and also tended to increase the frequency of CD27$^+$ memory B cells in MS remission, but without statistical significance. Consequently, the frequency of CD27$^+$ memory B cells after the dual stimulation was significantly lower in MS than HS, regardless of disease phase (31% in HS, 17% in MS remission, $p = 0.023$; 7.4% at MS relapse, $p = 0.0005$).

We further evaluated the frequency of IL-10-producing CD27$^-$ naïve and CD27$^+$ memory B cells in all CD19$^+$ B cells (Fig. 2D and E). The frequency of IL-10-producing CD27$^-$ naive B cells in CD19$^+$ B cells did not differ significantly among the three groups after TLR4 stimulation, whereas the frequency of IL-10-producing CD27$^+$ memory B cells in CD19$^+$ B cells was significantly lower in MS remission than HS (0.44% in HS, 0.12% in MS remission, $p = 0.0023$). After CD40 stimulation, the frequencies of IL-10-producing CD27$^-$ naïve and CD27$^+$ memory B cells in the CD19$^+$ B cell population were significantly lower in MS than HS, regardless of disease phase (CD27$^-$ naïve: 1.7% in HS, 1.1% in MS remission, $p = 0.019$; 0.57% at MS relapse, $p = 0.021$; CD27$^+$ memory: 0.96% in HS, 0.11% in MS remission, $p = 0.0001$; 0.19% at MS relapse, $p = 0.009$). After the dual stimulation of TLR4 and CD40, the frequency of IL-10-producing CD27$^-$ naïve B cells in the total CD19$^+$ B cells at MS relapse was significantly higher than that in MS remission and tended to be higher than in HS, but without statistical significance (2.9% in HS, 1.6% in MS remission, 6.2% at MS relapse, $p = 0.026$). The frequency of IL-10-producing CD27$^+$ memory B cells in the CD19$^+$ B cell population was significantly higher in HS than MS remission, but there was no significant difference between HS and MS relapse (1.4% in HS, 0.41% in MS remission, $p = 0.007$; 1.3% at MS relapse).

We hypothesized that fluorescence intensity (FI) of IL-10 positively correlated with their IL-10 production quantity of each IL-10-producing cells. Hence, to estimate IL-10 production after dual stimulation of TLR4 and CD40, we evaluated the mean FI (MFI) of IL-10 of B cells gated by IL-10-positivity. The MFI gated by IL-10 positivity did not differ significantly between CD19$^+$, CD27$^-$ naïve, and CD27$^+$ memory B cells in all the three groups (Fig. 3A). To further confirm that the frequency of IL-10 producing cells reflected their IL-10 production quantity, we evaluated the IL-10 production quantity by purified B cells after dual stimulation of TLR4 and CD40. There was a significant positive correlation between the frequency of IL-10-producing B cells and total amount of IL-10 production ($p = 0.016$, $R^2 = 0.588$) (Fig. 3B). These results

highly suggest that the frequency of IL-10-producing B cells positively correlates with total amount of IL-10 production by B cells.

Since it is very hard technically to isolate sufficient number of B cells from limited volume of peripheral blood derived from MS patients, we analyzed IL-10 production quantity by PBMC after dual stimulation of TLR4 and CD40. As in the case of the frequency of IL-10-producing B cells (Fig. 1A), we also found that IL-10 production by PBMC was lower in MS remission than HS and MS relapse, but the small number analysis did not provide statistical significance to the results (69 pg/ml in HS, n = 6, 36 pg/ml in MS remission, n = 6, 82.3 pg/ml at MS relapse, n = 4) (Fig. 3C).

3.5. Expression of TLR4 and CD40 on B cells in MS patients

The MFI of TLR4 on CD19$^+$ B cells was significantly lower in MS remission than HS and MS relapse (47.9 in MS remission, 57.1 in HS, $p = 0.0041$; 55.6 at MS relapse, $p = 0.0029$) (Fig. 4A). This outcome was mainly because of differences in CD27$^-$ naïve B cells since MFI of TLR4 on CD27$^+$ memory B cells did not differ significantly among the three groups. MFI of CD40 also did not differ significantly among the three groups (Fig. 4C). This was the case for both CD27$^-$ naïve and CD27$^+$ memory B cells.

We next evaluated the relationship between the frequency of IL-10-producing B cell subsets and MFI of either TLR4 or CD40 at baseline using Spearman correlation coefficients. There was no significant correlation found between these variables in both HS and MS (Table 2).

3.6. TLR- and CD40-mediated pro-inflammatory cytokine production by B cells in MS patients

The frequency of TNF-α-producing cells within the CD19$^+$ B cell population was significantly higher in MS remission (but not at relapse) than HS after TLR4 stimulation (6.7% in HS; 12.6% in MS remission, $p = 0.012$; 9.8% at MS relapse) (Supplementary Table 1). There were no other significant differences in the frequency of pro-inflammatory cytokine-producing cells in CD19$^+$ B cells among the three groups after various stimulations.

3.7. Effects of IFN-β treatment on TLR- and CD40-mediated IL-10 production by B cells

We investigated the effects of INF-β on IL-10 production by B cells in MS remission (Fig. 5A). The frequency of IL-10-producing cells in the total CD19$^+$ B cells was significantly higher in IFN-β responders than non-treated MS but only after CD40 stimulation (3.7% in responders, n = 7; 0.69% in non-treated, n = 7, $p = 0.025$). It was also higher in IFN-β responders than non-responders (0.69%, n = 4), but without statistical significance probably because of the small sample size. The frequency of CD27$^+$ memory B cells in CD19$^+$ B cells was lower in IFN-β responders than the other groups, without statistical significance (9.6% in non-treated, n = 6, 5.1% in responders, n = 4, 9.9% in non-responders, n = 3) (Fig. 5B). After CD40 stimulation, both the frequencies of IL-10-producing cells in CD27$^-$ naïve B cells (Fig. 5C) and IL-10-producing naïve B cells within the CD19$^+$ B cell population (Fig. 5D) was higher in IFN-β responders than the other groups, but these results did not reach

after dual stimulation of TLR4 and CD40 or TLR9 and CD40 in the indicated groups. Intra-individual frequency analysis of L-10-positive cells in CD19$^+$ B cells after dual stimulation of (C) TLR4 and CD40 or (D) TLR9 and CD40 were performed both at relapse and in remission in six patients with multiple sclerosis. *P* values were calculated using Kruskal–Wallis test and Mann-Whitney test (A). The Wilcoxon paired rank test was used for analysis of (C) and (D). HS = healthy participants; MSrem = multiple sclerosis in remission; MSrel = multiple sclerosis at relapse; TLR = toll-like receptor.

A

B

$$y = 31.66x + 4.296$$
$$R^2 = 0.588$$
$$P = 0.016$$

C
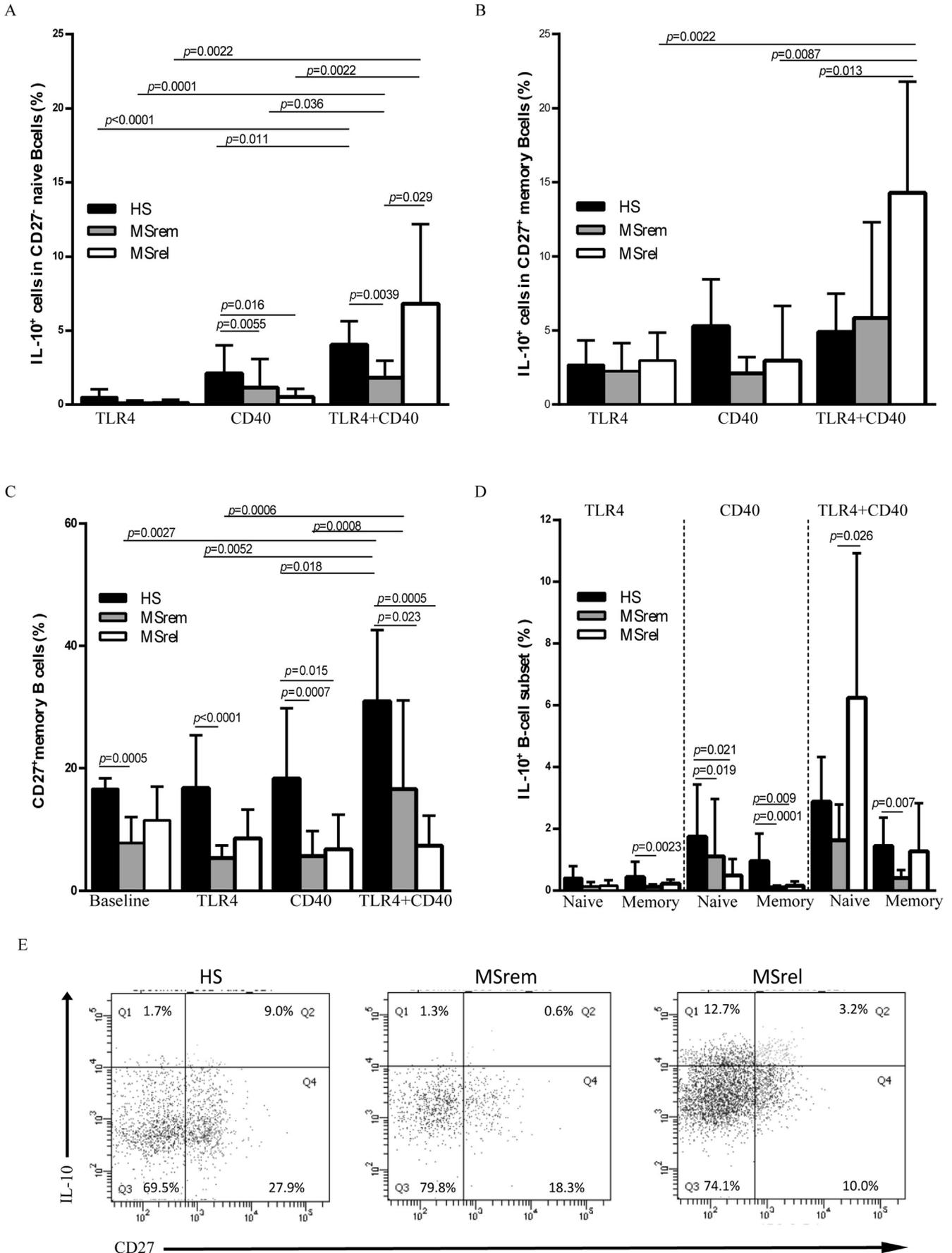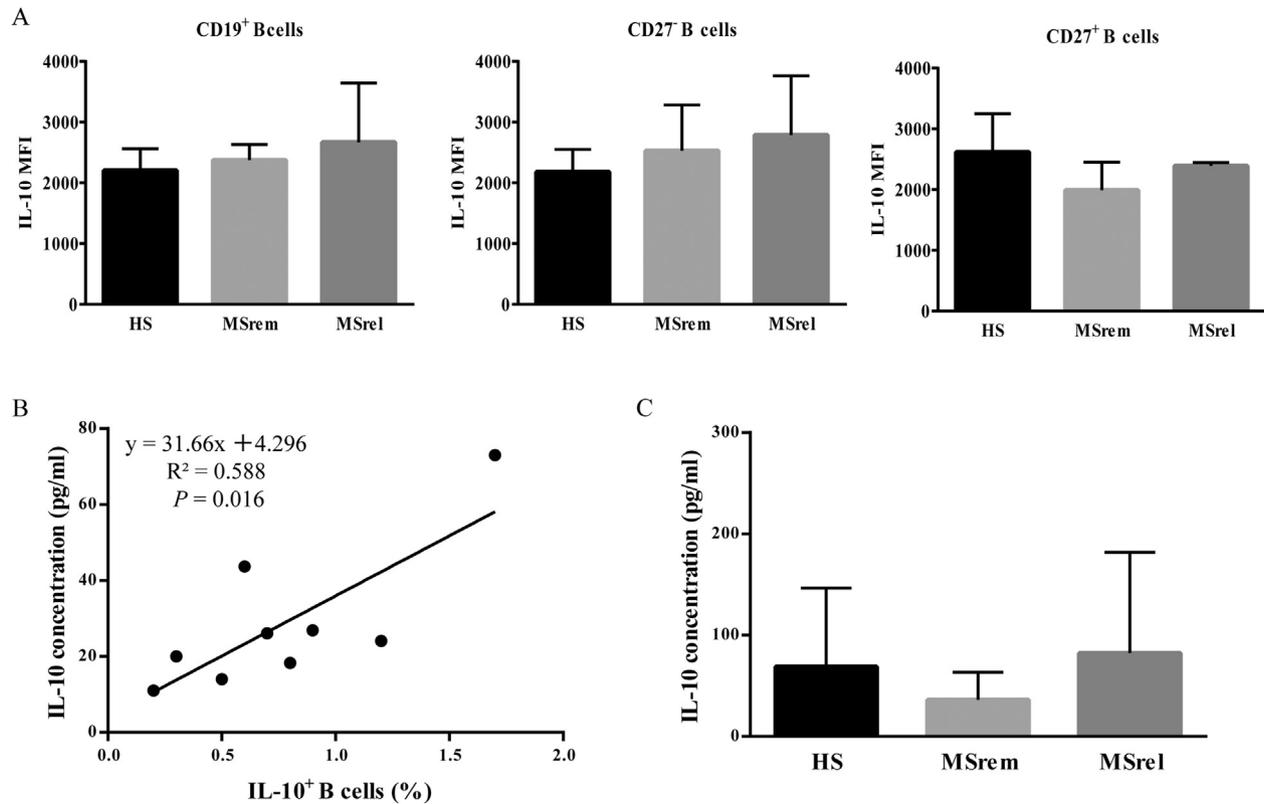
Fig. 3. IL-10 production after stimulation of TLR4 plus CD40. Peripheral blood mononuclear cells were stimulated through TLR4 plus CD40 and stained for surface CD19 and CD27, and intracellular IL-10. Mean fluorescence intensity (MFI) was analyzed in IL-10-expressing B cells. (A) Bar graphs show MFI (±standard deviation) of IL-10 gated by its positivity in CD19+ (total B cells), CD19+ and CD27− (naïve B cells), and CD19+ and CD27+ cells (memory B cells) in the indicated groups. (B) Frequency of IL-10-producing B cells positively correlated with total amount of IL-10 production. Purified B cells were stimulated through TLR4 plus CD40. Cells were stained for intracellular IL-10. IL-10 concentration in the supernatant was measured by Cytometric Beads Array Enhanced Sensitivity Flex Set System. (C) IL-10 production by Peripheral blood mononuclear cells after dual stimulation of TLR4 and CD40. Peripheral blood mononuclear cells were stimulated through TLR4 plus CD40. IL-10 concentration in the supernatant was measured by Cytometric Beads Array Enhanced Sensitivity Flex Set System. Bar graphs show mean (±standard deviation) concentration of IL-10 in the indicated groups. *P* values were calculated using linear regression analysis (B). HS = healthy participants; MSrem = multiple sclerosis in remission; MSrel = multiple sclerosis at relapse; TLR = toll-like receptor.

statistical significance (Fig. 5C; 0.6% in non-treated, 2.0% in responders, 1.0% in non-responders, Fig. 5D; 0.6% in non-treated, 2.0% in responders, 0.9% in non-responders).

4. Discussion

The main new findings of this study are: (1) the frequency of IL-10-producing cells in TLR4-stimulated B cells did not differ significantly between HS and MS, regardless of disease phase; (2) dual stimulation of TLR4 and CD40 synergistically increased the frequency of IL-10-producing B cells in both naïve and memory B cells at MS relapse; and (3) the frequency of IL-10-producing cells in CD40-stimulated B cells was significantly higher in IFN-β responders than non-treated MS patients.

Besides the harmful functions of pro-inflammatory cytokines, B cells can exert regulatory functions through IL-10 in the

pathogenesis of MS. Previous studies have shown that B cells from MS patients are deficient in their capacity to produce IL-10 after either TLR9 or CD40 stimulation [8—10]. Consistent with these results, we found the frequency of IL-10-producing cells in both TLR9- and CD40-stimulated B cells decreased significantly in MS compared with HS, regardless of disease phase.

In contrast to TLR9, the role of TLR4 signaling in the regulation of IL-10 production by B cells has not been studied in MS. However, epidemiological studies and exploratory clinical trials suggest beneficial effects of TLR4 signaling in MS. Epidemiological studies revealed an inverse correlation between MS prevalence and microbial infection containing TLR4 agonists such as *Trichuris trichiura* and *Mycobacterium tuberculosis* [25,26]. Furthermore, clinical trials of *Trichuris suis* ova and the *Mycobacterium bovis* strain Bacillus Calmette-Guerin vaccine showed favorable effects on MS disease activity [19—21,27,28]. These findings prompted us

Fig. 2. Frequency of IL-10-producing B cell subset after stimulation of TLR4, CD40, and TLR4 plus CD40. Peripheral blood mononuclear cells were stimulated through TLR4, CD40, and TLR4 plus CD40. Cells were stained for surface CD19, CD27, and intracellular IL-10. CD27 expression was used to divide naive and memory B cells. Bar graphs show mean (±standard deviation) frequencies of IL-10-positive cells in (A) CD19+ and CD27− naïve and (B) CD19+ and CD27+ memory B cells determined by flow cytometry in the indicated groups. (C) Bar graphs show mean (±standard deviation) frequencies of CD27+ cells (memory B cells) in CD19+ B cells before and after stimulation determined by flow cytometry in the indicated groups. (D) Bar graphs show mean (±standard deviation) frequencies of IL-10-positive CD27− (naïve B cells) and CD27+ cells (memory B cells) in CD19+ B cells determined by flow cytometry in the indicated groups. (E) Representative analysis of CD27 and IL-10 expression, gated on CD19+ B cells after dual stimulation of TLR4 and CD40 in the indicated groups. *P* values were calculated using Kruskal—Wallis test and Mann-Whitney test. HS = healthy participants; MSrem = multiple sclerosis in remission; MSrel = multiple sclerosis at relapse; TLR = toll-like receptor.
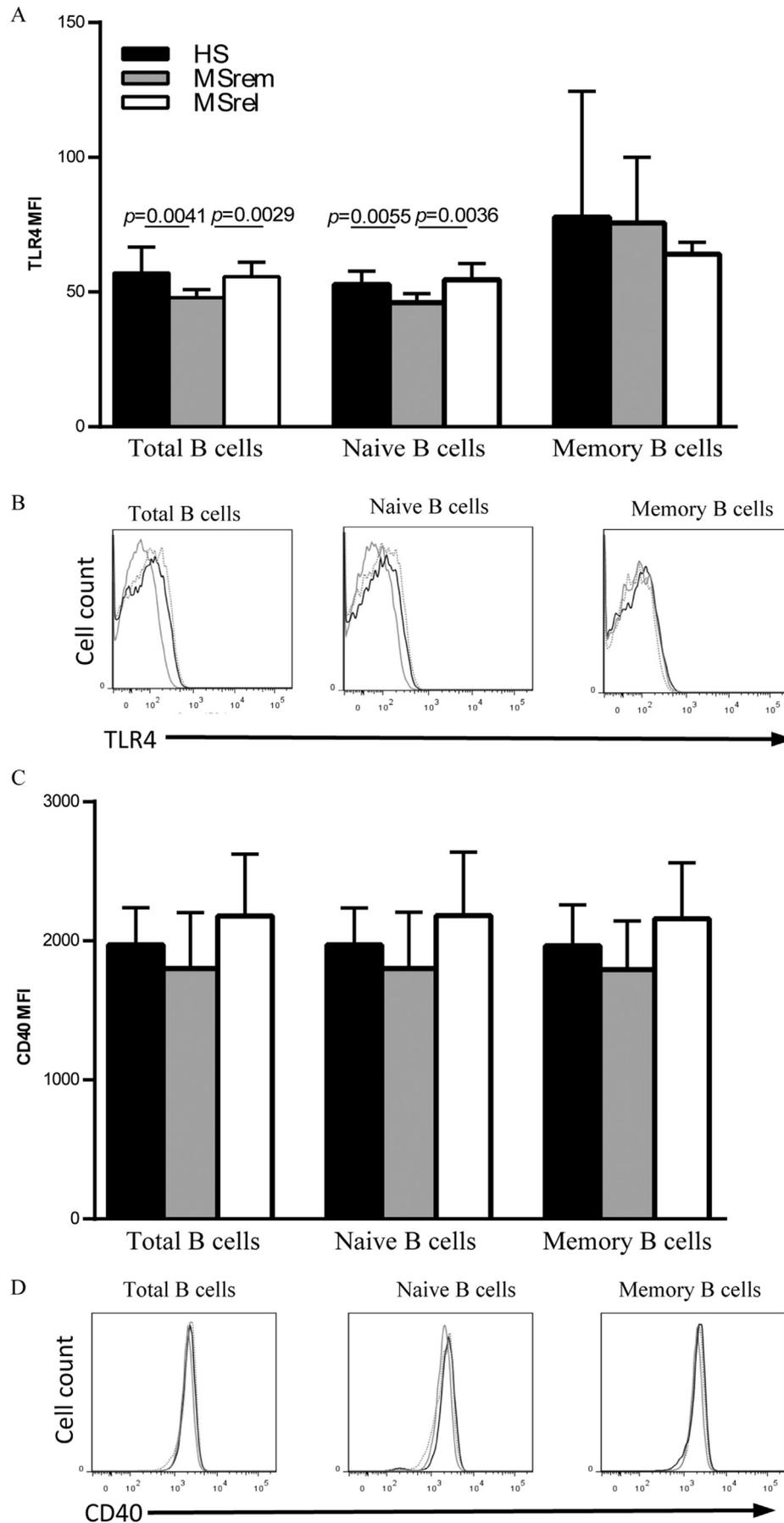
Table 2
Correlations between the frequency of IL-10-producing cells in indicated B cells and mean fluorescence intensity of TLR4 and CD40 at baseline.

Characteristics	Healthy Participants (n = 10)	Multiple Sclerosis (n = 20)
TLR4		
CD19$^+$ B cells	0.25 (0.49)	0.20 (0.42)
CD27$^-$ naïve B cells	0.38 (0.28)	0.23 (0.34)
CD27$^+$ memory B cells	0.37 (0.29)	0.073 (0.77)
CD40		
CD19$^+$ B cells	0.32 (0.36)	0.45 (0.078)
CD27$^-$ naïve B cells	0.33 (0.35)	0.31 (0.20)
CD27$^+$ memory B cells	0.26 (0.47)	0.43 (0.06)

Data are expressed as Spearman correlation coefficients: r (p-values).

to evaluate the ability of MS B cells to produce IL-10 in response to TLR4 stimulation. We found that IL-10 production in response to TLR4 stimulation was similar between MS patients and HS, regardless of disease phase, and further increased during relapse in the presence of CD40 co-stimulation without enhancing pro-inflammatory cytokine production. These findings align with previous studies reporting that CD40 stimulation augmented continuous IL-10 production by human B cells in response to TLR stimulation [7,29], and CD40 as well as TLR4 signaling was involved in B cell-mediated recovery from EAE through IL-10 production [5,18]. Taken together, our results indicate that cross-talk between TLR4 and CD40 signaling prompts B cells to exert regulatory functions during MS relapse.

Up-regulation of endogenous TLR4 ligands by heat shock protein and high mobility group box protein 1 has been demonstrated in both peripheral blood and active lesions of MS [30–33]. Through TLR4 signaling, heat shock protein 60-activated B cells produce IL-10 and down-regulate pro-inflammatory responses [34]. Assuming activated T cells provide CD40 ligands *in vivo*, B cells activated by endogenous TLR4 ligands may continue producing IL-10 through cognate interactions with T cells thereby limiting inflammatory T cell responses and facilitating recovery from a relapse.

The synergistic effect of dual stimulation of TLR4 and CD40 in increasing the frequency of IL-10-producing B cells at MS relapse was observed in both CD27$^-$ naïve and CD27$^+$ memory B cells. However, this effect was more pronounced in CD27$^-$ naïve B cells because of the lower frequency of CD27$^+$ memory B cells at MS relapse. Although both naïve and memory B cells produce IL-10, their roles in the pathogenesis of MS are thought to be different. IL-10-producing naïve B cells presumably play a role in preventing disease exacerbations, whereas IL-10-producing memory B cells play a role in resolving them [10,35]. Our results expand this hypothesis and suggest that IL-10-producing naïve B cells also play a role in promoting recovery from relapse via cross-talk between TLR4 and CD40 signaling. Interestingly, although the frequency of IL-10-producing cells in CD27$^+$ memory B cells did not differ significantly after dual stimulation of TLR4 and CD40 between HS and MS remission, its frequency in CD27$^-$ naïve B cells was significantly lower in MS remission than HS. This result might align with the hypothesis that IL-10-producing naïve B cells prevent inflammatory responses in autoimmunity [10,35]. However, it is not clear at present whether the capacity of naïve B cells to produce IL-10 in response to TLR4 and CD40 stimulation correlate with disease activity. Large scale of intra-individual longitudinal study or further studies in patients with different disease activity are needed.

TLR4 but not CD40 expression on naïve B cells was significantly lower in MS remission than HS and MS relapse. However, there was no correlation between TLR4 expression and the frequency of IL-10-producing naïve B cells after either TLR4 stimulation or dual stimulation of TLR4 and CD40. Further study is needed to clarify the intracellular signaling pathway that controls IL-10 production by B cells following TLR4 and CD40 stimulation.

IFN-β has been shown to have beneficial effects on MS disease activity. However, a significant proportion of patients do not respond to IFN-β. In this study, we could not find any effect of IFN-β on B cell capacity to produce IL-10 following TLR4 stimulation. However, we found that CD40-mediated IL-10 production by B cells increased in IFN-β responders, and naïve B cells represented the major proportion of IL-10-producing B cells. This result aligns with previous studies showing that increased IL-10 production is one of the hallmarks of the therapeutic effects of IFN-β [36–38] and naïve B cells play a regulatory role in disease remission [10,35]. This may be interpreted as B cells interacting with activated T cells through the CD40–CD40 ligand pathway dampening further activation of T cells through provision of IL-10 in IFN-β responders, thereby preventing clinical relapse.

In conclusion, we found that TLR4 and TLR9 signaling have distinct roles in regulating IL-10 production by B cells in MS. TLR9-mediated IL-10 production was dysregulated, while TLR4-mediated IL-10 production was restored to normal levels in MS and further increased at relapse in the presence of CD40 signaling in the context of PBMC. The results may be due to altered cytokine milieu by other immune cells expressing TLR4 and/or TLR9. Further study is needed to clarify the interplay between B cells and other immune cells. In addition, molecular mechanisms behind the cross-talk between TLR4 and CD40 that controls IL-10 production by B cells should be elucidated. Another interesting finding is that CD40 signaling in B cells is involved in the response to IFN-β treatment. Collectively, regulation of TLR4 and CD40 signaling in B cells may be a promising novel approach for MS therapy.

Fig. 4. Expression levels of TLR4 and CD40 on B cells. Peripheral blood mononuclear cells were stained for surface CD19, CD27, CD40, and TLR4. Expression intensity of cell surface TLR4 and CD40 was determined by mean fluorescence intensity (MFI). (A) Bar graphs show MFI (±standard deviation) of TLR4 on CD19$^+$ (total B cells), CD19$^+$ and CD27$^-$ (naïve B cells), and CD19$^+$ and CD27$^+$ cells (memory B cells) in the indicated groups. (B) Representative histograms of TLR4 expression on total B cells, naïve B cells and memory B cells in HS (black lines), MSrem (gray lines) and MSrel (gray dotted lines). (C) Bar graphs show MFI (±standard deviation) of CD40 on total B cells, naïve B cells, and memory B cells in the indicated groups. (D) Representative histograms of CD40 expression on total B cells, naïve B cells and memory B cells in HS (black lines), MSrem (gray lines) and MSrel (gray dotted lines). P values were calculated using Kruskal–Wallis test and Mann-Whitney test (A, C). HS = healthy participants; MSrem = multiple sclerosis in remission; MSrel = multiple sclerosis at relapse; TLR = toll-like receptor.
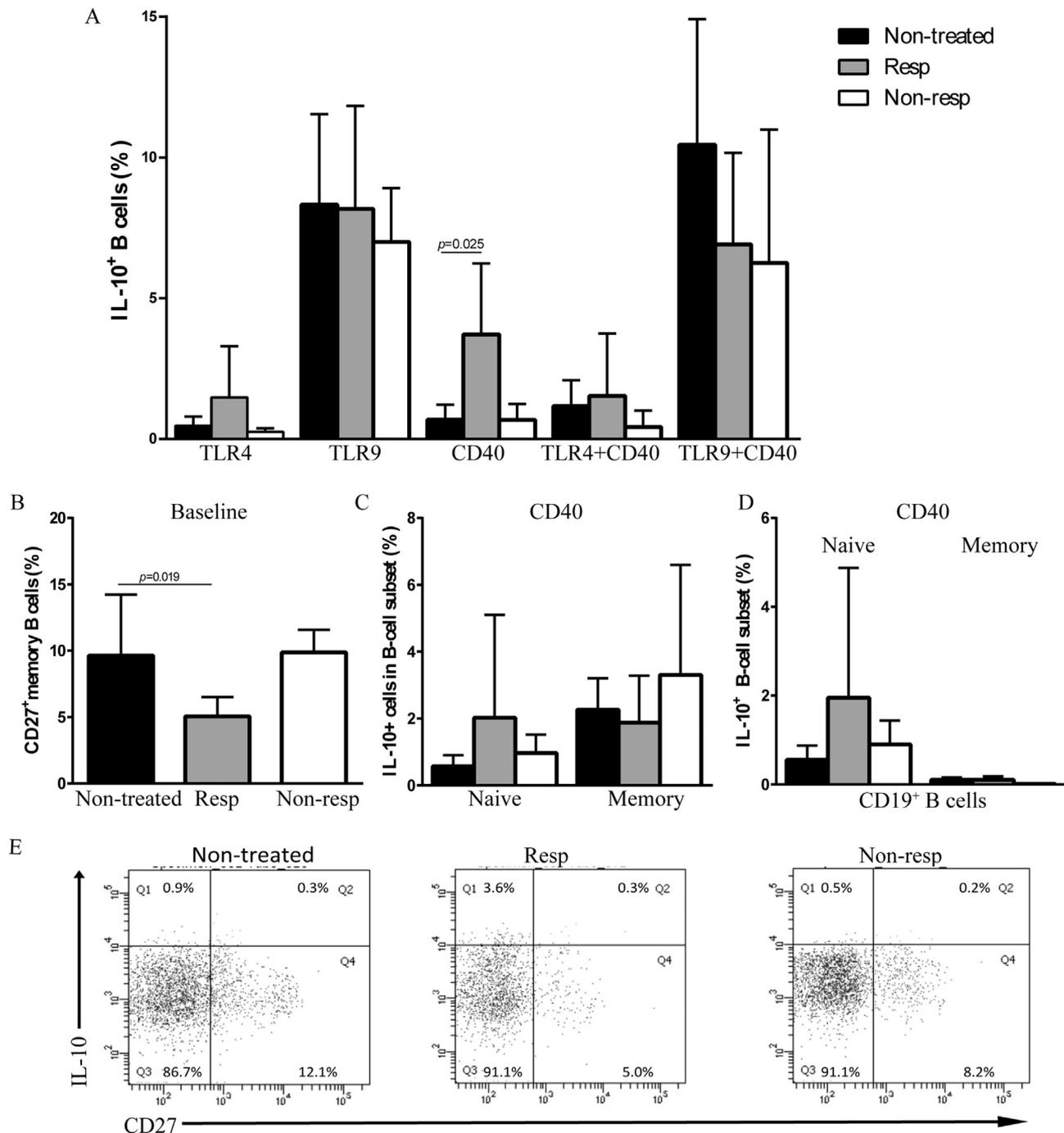
Fig. 5. Effects of IFN-β treatment on TLR- and CD40-mediated IL-10 production. Peripheral blood mononuclear cells from patients with multiple sclerosis were stimulated through TLR and CD40 as indicated. Cells were stained for surface CD19 and CD27 and intracellular IL-10. (A) Bar graphs show mean (±standard deviation) frequencies of IL-10-positive cells in CD19$^+$ B cells determined by flow cytometry in the indicated groups. (B) Bar graphs show mean (±standard deviation) frequencies of CD27$^+$ cells (memory B cells) in CD19$^+$ B cells before stimulation as determined by flow cytometry in the indicated groups. (C) Bar graphs show mean (±standard deviation) frequencies of IL-10-positive cells in CD19$^+$ and CD27$^-$ naïve and CD19$^+$ and CD27$^+$ memory B cells determined by flow cytometry in the indicated groups. (D) Bar graphs show mean (±standard deviation) frequencies of IL-10-positive CD27$^-$ (naïve B cells) and CD27$^+$ cells (memory B cells) in CD19$^+$ B cells determined by flow cytometry in the indicated groups. (E) Representative analysis of CD27 and IL-10 expression gated on CD19. The frequencies of CD27$^-$ IL-10$^+$ (upper left), CD27$^-$ IL-10$^-$ (lower left), CD27$^+$IL-10$^+$ (upper right) and CD27$^+$IL-10$^-$ (lower right) cells in CD19$^+$ B cells after single stimulation of CD40 is shown in the indicated groups. *P* values were calculated using Kruskal–Wallis test and Mann-Whitney test. IFN-β = interferon-β; Resp = IFN-β responders; Non-resp = IFN-β non-responders; TLR = toll-like receptor.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2017.10.011.

References

[1] M. Sospedra, R. Martin, Immunology of multiple sclerosis, Annu. Rev. Immunol. 23 (2005) 683–747.

[2] S.L. Hauser, E. Waubant, D.L. Arnold, T. Vollmer, J. Antel, R.J. Fox, et al., B-cell depletion with rituximab in relapsing-remitting multiple sclerosis, N. Engl. J. Med. 358 (2008) 676–688.

[3] L. Kappos, D. Li, P.A. Calabresi, P. O'Connor, A. Bar-Or, F. Barkhof, et al., Ocrelizumab in relapsing-remitting multiple sclerosis: a phase 2, randomised, placebo-controlled, multicentre trial, Lancet 378 (2011) 1779–1787.

[4] P.S. Sorensen, R. Lisby, R. Grove, F. Derosier, S. Shackelford, E. Havrdova, et al., Safety and efficacy of ofatumumab in relapsing-remitting multiple sclerosis: a phase 2 study, Neurology 82 (2014) 573–581.

[5] S. Fillatreau, C.H. Sweenie, M.J. McGeachy, D. Gray, S.M. Anderton, B cells regulate autoimmunity by provision of IL-10, Nat. Immunol. 3 (2002) 944–950.

[6] S.D. Wolf, B.N. Dittel, F. Hardardottir, C.A. Janeway Jr., Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice, J. Exp. Med. 184 (1996) 2271–2278.

[7] Y. Iwata, T. Matsushita, M. Horikawa, D.J. Dilillo, K. Yanaba, G.M. Venturi, et al., Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells, Blood 117 (2011) 530–541.

[8] M. Hirotani, M. Niino, T. Fukazawa, S. Kikuchi, I. Yabe, S. Hamada, et al., Decreased IL-10 production mediated by Toll-like receptor 9 in B cells in multiple sclerosis, J. Neuroimmunol. 221 (2010) 95–100.

[9] M. Duddy, M. Niino, F. Adatia, S. Hebert, M. Freedman, H. Atkins, et al., Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis, J. Immunol. 178 (2007) 6092–6099.

[10] S. Knippenberg, E. Peelen, J. Smolders, M. Thewissen, P. Menheere, J.W. Cohen Tervaert, et al., Reduction in IL-10 producing B cells (Breg) in multiple sclerosis is accompanied by a reduced naive/memory Breg ratio during a relapse but not in remission, J. Neuroimmunol. 239 (2011) 80–86.

[11] A.J. Steelman, Infection as an environmental trigger of multiple sclerosis disease exacerbation, Front. Immunol. 6 (2015) 520.

[12] R. Medzhitov, P. Preston-Hurlburt, C.A. Janeway Jr., A human homologue of the Drosophila Toll protein signals activation of adaptive immunity, Nature 388 (1997) 394–397.

[13] C.R. Ruprecht, A. Lanzavecchia, Toll-like receptor stimulation as a third signal required for activation of human naive B cells, Eur. J. Immunol. 36 (2006) 810–816.

[14] G. Liu, L. Zhang, Y. Zhao, Modulation of immune responses through direct activation of Toll-like receptors to T cells, Clin. Exp. Immunol. 160 (2010) 168–175.

[15] P. Velupillai, D.A. Harn, Oligosaccharide-specific induction of interleukin 10 production by B220+ cells from schistosome-infected mice: a mechanism for regulation of CD4+ T-cell subsets, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 18–22.

[16] J. Correale, M. Farez, G. Razzitte, Helminth infections associated with multiple sclerosis induce regulatory B cells, Ann. Neurol. 64 (2008) 187–199.

[17] J. Correale, M. Farez, Association between parasite infection and immune responses in multiple sclerosis, Ann. Neurol. 61 (2007) 97–108.

[18] V. Lampropoulou, K. Hoehlig, T. Roch, P. Neves, E.C. Gomez, C.H. Sweenie, et al., TLR-activated B cells suppress T cell-mediated autoimmunity, J. Immunol. 180 (2008) 4763–4773.

[19] A. Paolillo, M.G. Buzzi, E. Giugni, U. Sabatini, S. Bastianello, C. Pozzilli, et al., The effect of Bacille Calmette-Guerin on the evolution of new enhancing lesions to hypointense T1 lesions in relapsing remitting MS, J. Neurol. 250 (2003) 247–248.

[20] G. Ristori, M.G. Buzzi, U. Sabatini, E. Giugni, S. Bastianello, F. Viselli, et al., Use of bacille calmette-guerin (BCG) in multiple sclerosis, Neurology 53 (1999) 1588–1589.

[21] G. Ristori, S. Romano, S. Cannoni, A. Visconti, E. Tinelli, L. Mendozzi, et al., Effects of Bacille Calmette-Guerin after the first demyelinating event in the CNS, Neurology 82 (2014) 41–48.

[22] P.A. Blair, K.A. Chavez-Rueda, J.G. Evans, M.J. Shlomchik, A. Eddaoudi, D.A. Isenberg, et al., Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice, J. Immunol. 182 (2009) 3492–3502.

[23] C.H. Polman, S.C. Reingold, B. Banwell, M. Clanet, J.A. Cohen, M. Filippi, et al., Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria, Ann. Neurol. 69 (2011) 292–302.

[24] T. Decker, F. Schneller, T. Sparwasser, T. Tretter, G.B. Lipford, H. Wagner, et al., Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells, Blood 95 (2000) 999–1006.

[25] E. Andersen, H. Isager, K. Hyllested, Risk factors in multiple sclerosis: tuberculin reactivity, age at measles infection, tonsillectomy and appendectomy, Acta Neurol. Scand. 63 (1981) 131–135.

[26] J.O. Fleming, T.D. Cook, Multiple sclerosis and the hygiene hypothesis, Neurology 67 (2006) 2085–2086.

[27] J.O. Fleming, A. Isaak, J.E. Lee, C.C. Luzzio, M.D. Carrithers, T.D. Cook, et al., Probiotic helminth administration in relapsing-remitting multiple sclerosis: a phase 1 study, Mult. Scler. 17 (2011) 743–754.

[28] F. Benzel, H. Erdur, S. Kohler, M. Frentsch, A. Thiel, L. Harms, et al., Immune monitoring of Trichuris suis egg therapy in multiple sclerosis patients, J. Helminthol. 86 (2012) 339–347.

[29] S.M. Anderton, S. Fillatreau, Activated B cells in autoimmune diseases: the case for a regulatory role, Nat. Clin. Pract. Rheumatol. 4 (2008) 657–666.

[30] M.J. Mansilla, M. Comabella, J. Rio, J. Castillo, M. Castillo, R. Martin, et al., Upregulation of inducible heat shock protein-70 expression in multiple sclerosis patients, Autoimmunity 47 (2014) 127–133.

[31] M. Gambuzza, N. Licata, E. Palella, D. Celi, V. Foti Cuzzola, D. Italiano, et al., Targeting Toll-like receptors: emerging therapeutics for multiple sclerosis management, J. Neuroimmunol. 239 (2011) 1–12.

[32] S. Malhotra, N. Fissolo, M. Tintore, A.C. Wing, J. Castillo, A. Vidal-Jordana, et al., Role of high mobility group box protein 1 (HMGB1) in peripheral blood from patients with multiple sclerosis, J. Neuroinflam. 12 (2015) 48.

[33] A. Andersson, R. Covacu, D. Sunnemark, A.I. Danilov, A. Dal Bianco, M. Khademi, et al., Pivotal advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis, J. Leukoc. Biol. 84 (2008) 1248–1255.

[34] M. Cohen-Sfady, G. Nussbaum, M. Pevsner-Fischer, F. Mor, P. Carmi, A. Zanin-Zhorov, et al., Heat shock protein 60 activates B cells via the TLR4-MyD88 pathway, J. Immunol. 175 (2005) 3594–3602.

[35] A. Rieger, A. Bar-Or, B-cell-derived interleukin-10 in autoimmune disease: regulating the regulators, Nat. Rev. Immunol. 8 (2008) 486–487.

[36] P.B. Carrieri, P. Ladogana, G. Di Spigna, M.F. de Leva, M. Petracca, S. Montella, et al., Interleukin-10 and interleukin-12 modulation in patients with relapsing-remitting multiple sclerosis on therapy with interferon-beta 1a: differences in responders and non responders, Immunopharmacol. Immunotoxicol. 30 (2008) 1–9.

[37] R.A. Rudick, R.M. Ransohoff, R. Peppler, S. VanderBrug Medendorp, P. Lehmann, J. Alam, Interferon beta induces interleukin-10 expression: relevance to multiple sclerosis, Ann. Neurol. 40 (1996) 618–627.

[38] M. Kvarnstrom, J. Ydrefors, C. Ekerfelt, M. Vrethem, J. Ernerudh, Longitudinal interferon-beta effects in multiple sclerosis: differential regulation of IL-10 and IL-17A, while no sustained effects on IFN-gamma, IL-4 or IL-13, J. Neurol. Sci. 325 (2013) 79–85.