fingolimod, as indicated by their reduction in the peripheral blood following fingolimod treatment. It is demonstrated in mice that surface expression levels of S1P1 on B cells in the SLT are controlled by transcription levels and CD69-mediated internalisation of S1P1. Stimulation of B-cell receptors induces not only a cessation of S1P1 transcription, but also an upregulation of CD69. Both of these changes reduce the expression levels of surface S1P1 in the SLT to some extent.[2]

Although we were not able to directly analyse B cells in the SLT of the patients, we speculated that surface S1P1 expression on mBs within the SLT in human may also decrease greatly, following antigen activation and exposure to fingolimod, which would result in these B lymphocytes having a reduced responsiveness to S1P. In fact, the activated mB subpopulations that we isolated from the patients' peripheral blood, in particular $CD38^{high}$ mB, were found to contain a substantial proportion of $Ki\text{-}67^+$ cells (Figure 3(a) and (b)). We confirmed that the proportions of $Ki\text{-}67^+$ cells in the activated $CD38^{int}$ and $CD38^{high}$ mB subpopulations were significantly decreased following fingolimod treatment, suggesting that recently-activated cells were selectively trapped in the SLT following fingolimod treatment. Because activation of autoreactive mBs in the SLT followed by their migration to the CNS could trigger a relapse of RRMS,[35] we assumed that inhibition of activated mB cell egress from the SLT was at least partly involved in the reduced relapses of RRMS after fingolimod treatment.

We also identified a PB subpopulation that is relatively resistant to fingolimod as being $CD138^+$ PBs. The frequency of the $CD138^+$ subpopulation in the total PBs, and that of $CXCR3^+$ cells in $CD138^+$ PBs, was significantly increased by fingolimod treatment. Of note, the $CD138^+CXCR3^+$ PBs are enriched in the CSF of NMO during relapse,[27] and fingolimod could induce exacerbation of NMO, accompanied by the appearance of large brain lesions.[11,12] Although knowledge on the biology of PBs is limited, the percentages of $CCR7^+$ cells are much lower as compared with nBs or mBs, indicating that fingolimod may differentially alter the in vivo migration of PBs and other B cells.

It is of relevance to note that despite reductions of circulating lymphocytes, RRMS patients receiving fingolimod may develop clinical relapses. These relapses are not always mild, but could be serious and accompany huge brain lesions.[7–10] Although the trapping of regulatory lymphocytes in the SLT[8,9] or the enrichment for $CD45RO^-CCR7^-CD8^+$ T cells in the CSF[7] is proposed as a possible mechanism for formation of tumefactive brain lesions, we were very curious to know if the increased proportion of $CD138^+$ PBs over other lymphocytes in the peripheral blood might influence the character of the CNS pathology and induce large demyelinating lesions. In fact, it was recently reported that $CD45^+CD19^+CD138^+$ PBs are relatively enriched in the CSF of fingolimod-treated MS patients,[16] raising the possibility that the dominance of $CD138^+$ PBs in the peripheral blood is preserved or even promoted in the CNS of patients with MS who develop tumefactive brain lesions[7–10] and NMO patients who deteriorate[11,12] after being treated with fingolimod. Therefore, resistance of activated PBs in fingolimod-treated patients with MS or NMO may give us a clue to understanding the individual patients' differences regarding the effectiveness of fingolimod therapy.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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RESEARCH ARTICLE Open Access

Apathy/depression, but not subjective fatigue, is related with cognitive dysfunction in patients with multiple sclerosis

Masaaki Niino[1*], Nobuhiro Mifune[2], Tatsuo Kohriyama[3], Masahiro Mori[4], Takashi Ohashi[5], Izumi Kawachi[6], Yuko Shimizu[7], Hikoaki Fukaura[8,9], Ichiro Nakashima[10], Susumu Kusunoki[11], Katsuichi Miyamoto[11], Kazuto Yoshida[12], Takashi Kanda[13], Kyoichi Nomura[9], Takashi Yamamura[14], Fumihito Yoshii[15], Jun-ichi Kira[16], Shunya Nakane[17], Kazumasa Yokoyama[18], Makoto Matsui[19], Yusei Miyazaki[20] and Seiji Kikuchi[20]

Abstract

Background: Cognitive impairment could affect quality of life for patients with multiple sclerosis (MS), and cognitive function may be correlated with several factors such as depression and fatigue. This study aimed to evaluate cognitive function in Japanese patients with MS and the association between cognitive function and apathy, fatigue, and depression.

Methods: The Brief Repeatable Battery of Neuropsychological tests (BRB-N) was performed in 184 Japanese patients with MS and 163 healthy controls matched for age, gender, and education. The Apathy Scale (AS), Fatigue Questionnaire (FQ), and Beck Depression Inventory Second Edition (BDI-II) were used to evaluate apathy, fatigue, and depression, respectively. Student's t-test was used to compare MS patients and healthy controls. Correlations between two factors were assessed using the Pearson correlation test, and multiple regression analysis was used to evaluate how much each factor affected the BRB-N score.

Results: In all BRB-N tests, patients with MS scored significantly lower than controls, and the effect size of symbol digit modalities test was the highest among the 9 tests of the BRB-N. Patients with MS had higher AS ($p < 0.001$), FQ ($p < 0.0001$), and BDI-II ($p < 0.0001$) scores than controls. In patients with MS, scores on most of the BRB-N tests correlated with scores on the AS and BDI-II; however, there was little correlation between scores on the BRB-N tests and those on the FQ.

Conclusions: Cognitive function was impaired, particularly information-processing speed, and decreased cognitive function was correlated with apathy and depression in Japanese patients with MS. Despite the association between cognitive variables and depression/apathy, cognitive function was impaired beyond the effect of depression and apathy. However, subjective fatigue is not related with cognitive impairment. Taken together, this suggests that different therapeutic approaches are needed to improve subjective fatigue and cognition, and thereby quality of life, in patients with MS.

Keywords: Multiple sclerosis, Cognition, Apathy, Fatigue, Depression, Japanese

* Correspondence: niino@hok-mc.hosp.go.jp
[1]Department of Clinical Research, Hokkaido Medical Center, Yamanote 5jo 7chome, Nishi-ku, Sapporo 063-0005, Japan
Full list of author information is available at the end of the article

Background

The prevalence of cognitive dysfunction in multiple sclerosis (MS) has been historically underestimated due to difficulty in detecting cognitive impairment during brief office visits without performing a formal neuropsychological assessment and a widespread belief that cognitive dysfunction occurs rarely and then only in the advanced stages of the disease [1]. However, in neuropsychological studies, 40–65% of MS patients show cognitive impairment with prominent involvement of memory, sustained attention, and information processing speed [2]. Prevalence of cognitive dysfunction in MS varies among studies depending on the type of tests used and whether the studies are based in community or clinical settings, with clinical settings showing higher rates [3]. To evaluate cognitive deficits in MS, a focused measure of cognitive abilities using the Brief Repeatable Battery of Neuropsychological tests (BRB-N) was developed [4,5]. The BRB-N was originally written in English, and has been translated into other languages including Dutch [6] and Spanish [7]. Test scores on the BRB-N are influenced by variables such as age, gender, and level of education [6,8], and the BRB-N was shown to have a sensitivity of 71% and a specificity of 94% in discriminating MS patients with and without cognitive impairment [9].

Apathy has been defined as lack of motivation not attributable to diminished level of consciousness, cognitive impairment, or emotional distress, and the three domains of apathy are considered to be "deficits in goal-directed behavior", "a decrement in goal-related thought content", and "emotional indifference with flat affect" [10]. Fatigue is a frequent complication of MS, and MS patients often report that fatigue impairs their cognitive function. However, the relation between fatigue and cognitive performance is complex and inconsistent [11]. Depression is also a common symptom of MS, and recent studies suggest that information processing speed, working memory, and executive functioning of cognitive function may indeed be affected in patients with moderate to severe depression [12].

It is important for patient management to detect cognitive impairment accurately. Further, the relationship between cognitive impairment and the emergence of neuropsychiatric disorders in patients with MS remains unclear, and apathy, fatigue, and depression have not been investigated in Japanese patients with MS. The aim of this study was to evaluate cognitive function in Japanese patients with MS, and the association between cognitive function and fatigue, apathy, and depression.

Methods

Patients with MS and healthy individuals

This study was conducted between November 2010 and March 2012 with 184 Japanese patients with MS (female/male = 135/49) diagnosed using the 2005 revised McDonald criteria [13] at 18 sites (Hiroshima City Hospital, Chiba University, Tokyo Women's Medical University Yachiyo Medical Center, Niigata University, Tokyo Women's Medical University School of Medicine, Saitama Medical School, Tohoku University Graduate School of Medicine, Kinki University School of Medicine, Asahikawa Red Cross Hospital, Yamaguchi University Graduate School of Medicine, National Center of Neurology and Psychiatry, Tokai University School of Medicine, Kyushu University, Nagasaki Kawatana Medical Center, Iwate Medical University, Juntendo University School of Medicine, Kanazawa Medical University, and Hokkaido Medical Center) in Japan. Patients with neuromyelitis optica (NMO) or NMO spectrum disorders were excluded from this study. Patients were categorized according to MS subtype: 2 had primary progressive, 167 had relapsing–remitting, and 15 had secondary progressive disease, and did not experience relapses for at least 1 month before participating in this study. The mean age of the MS patients was 39.3 years (SD. 10.1; range 18–71 years). Duration of compulsory education in Japan is 9 years and the mean duration of education excluding compulsory education in this sample was 4.92 years (SD. 1.83; range 0–9 years). The mean age at onset was 30.0 years (SD. 10.1) and the mean duration of disease was 9.3 years (SD. 7.2). The mean Expanded Disability Status Scale (EDSS) was 2.38 (SD. 2.04; range 0–8.5). Among 184 patients with MS, 109 patients received interferon β (IFNβ) as disease modifying drugs (DMDs) when they participated in this study. Twenty-five patients received other DMDs such as fingolimod and natalizumab, and 50 patients did not receive any DMDs. A total of 163 healthy controls (female/male = 119/44) participated in this study. The mean age of the healthy controls was 39.2 years (SD. 11.9; range 19–76 years). The mean duration of education excluding compulsory education was 5.15 years (SD. 2.08; range 0–13 years). Differences in sex ratio, duration of education, and age at examination between the patients and controls were not significant ($p > 0.05$). People with diseases of the central nervous system or major medical illnesses were excluded from the healthy control group. All participants had adequate vision to complete testing. The study protocol was approved by the ethics committee of each participating site, and all patients and healthy controls gave their written informed consent to participate in the study.

Battery for neuropsychological evaluation, apathy, fatigue, and depressive state

Assessment of cognitive function

For neuropsychological evaluation, patients and healthy individuals completed the BRB-N, which includes tests of verbal learning and memory (selective reminding test, SRT), visuospatial memory and learning (10/36 spatial recall test, SPART), attention, information processing,

and working memory (paced auditory serial addition test, PASAT, and symbol digit modalities test, SDMT), and verbal fluency (word list generation test, WLG). The BRB-N, which was originally written in English, was translated into Japanese and used for assessment of neuropsychological functions. The test battery was administered in the following order: SRT, SPART, SDMT, PASAT, delayed recall of the SRT (SRT-D), delayed recall of the SPART (SPART-D), and WLG. Scores derived from these tests included long-term storage (SRT-LTS), consistent long-term retrieval (SRT-CLTR), and delayed recall (SRT-D) from the SRT, immediate recall (SPART) and delayed recall (SPART-D) from the SPART, total score from the SDMT, PASAT 2-second and 3-second versions (PASAT2 and PASAT3), and total score from the WLG test.

Assessment of apathy

Apathy was measured using the Apathy Scale (AS), which is an abridged version of an apathy scale designed by Robert Marin [14], with some modifications [15]. Briefly, patients were provided with four possible answers to 14 questions: "not at all", "slightly", "some", and "a lot". Each score ranged from 0 to 42 and higher scores indicated more severe apathy [15]. The AS was translated into Japanese and had been used previously in a study of Japanese patients with stroke [16].

Assessment of fatigue

In 1989, Krupp et al. reported data of fatigue in MS using the Fatigue Severity Scale [17]. The group expanded the scale of the Fatigue Questionnaire (FQ) and administered the FQ to a large group of medical and psychiatric patients [18]. The FQ, which was translated into Japanese and has been used previously [19], was used to measure fatigue in patients with MS. The FQ consists of 29 items each of which is a statement about fatigue and is rated from 1 representing "completely disagree" to 7 representing "completely agree", with a higher score indicating more fatigue [18]. Mean scores were calculated for each patient.

Assessment of depression

The Beck Depression Inventory second edition (BDI-II), which consists of 21 items rated on a scale from 0 to 3, is a valid and reliable measure of depressive state [20]. The Japanese version of the BDI-II, which was developed to be able to assess depressive symptoms in Japanese people, is psychometrically robust [21], and was used for evaluation of depression in the present study.

Statistical analysis

Statistical analyses were performed using the SAS 9.3 software package (SAS Institute Inc., Cary, NC). For analysis, raw data for the 9 tests (SRT-LTS, SRT-CLTR, SRT-D, SPART, SPART-D, SDMT, PASAT3, PASAT2, and WLG) were used, and scores for each of these tests were standardized as a mean score of 0 and standard deviation of 1. Student's t-test was used to compare average data between MS patients and healthy controls or between MS patients who received IFNβ and those who did not receive IFNβ. Correlations between two factors were assessed using the Pearson correlation test. Multiple regression analysis was used to evaluate how much each factor—patient, AS score, FQ score, and BDI-II score—affected the BRB-N score. p values less than 0.05 were considered statistically significant.

Results

BRB-N data in MS patients and healthy controls

Cronbach's alpha coefficients for all 9 BRB-N test scores were 0.93 in MS patients and 0.82 in the healthy control group, suggesting a high level of confidence. Thus, the BRB-N translated into Japanese showed a high internal consistency for each category and all scores. Table 1 shows mean BRB-N scores in MS patients and healthy controls. Table 2 shows a significant negative correlation between age at examination and each of the BRB-N components, except for WLG, was found in healthy controls. Negative correlations between duration of education and SRT-LTS, SRT-CTLR, SRT-D, SDMT, and PASAT2 were found in healthy controls, although there were no correlations between score and duration of education in the other 4 tests. In all 9 tests, scores were significantly lower in MS patients than in healthy controls. Table 2 also shows the standardized scores for each test in patients and healthy controls. To evaluate which test score is most different between patients and healthy controls, effect size (Cohen's d) was calculated. It was found that SDMT had the greatest effect size (1.34) of the 9 items (SRT-LTS, 0.67; SRT-CLTR, 0.72; SRT-D, 0.67; SPART, 0.86; SPART-D, 0.67; PASAT3, 0.95; PASAT2, 0.96; and WLG, 0.95). In the comparison of MS patients who received IFNβ and those who did not receive IFNβ, there were not any significant differences in all 9 BRB-N tests between the two groups.

Correlation of disease duration or EDSS with BRB-N in MS patients

Table 3 shows that in each of the 9 tests except the WLG, a significant but weak negative correlation was found between disease duration and score. On the other hand, relatively strong negative correlations were found between the EDSS and BRB-N scores in MS patients.

Apathy, fatigue, and depression in MS patients and healthy controls

Mean scores on the AS, FQ, and BDI-II in MS patients were 14.38 ± 6.98 (range, 0–34), 3.89 ± 1.18 (range,

Table 1 Mean BRB-N scores in patients with MS and healthy controls

BRB-N	MS patients		Healthy controls	
	Raw scores	Standardized scores	Raw scores	Standardized scores
SRT-LTS	40.85 ± 17.18 (0–72)	−0.30 ± 1.10	50.75 ± 11.68 (14–70)	0.34 ± 0.75
SRT-CLTR	31.43 ± 18.68 (0–72)	−0.32 ± 1.04	43.60 ± 14.58 (2–70)	0.36 ± 0.81
SRT-D	7.99 ± 3.07 (0–12)	−0.30 ± 1.13	9.71 ± 1.87 (5–12)	0.34 ± 0.69
SPART	18.91 ± 5.51 (5–30)	−0.37 ± 1.00	23.26 ± 4.55 (10–30)	0.42 ± 0.83
SPART-D	6.85 ± 2.34 (0–10)	−0.30 ± 1.05	8.26 ± 1.85 (1–12)	0.34 ± 0.83
SDMT	46.20 ± 15.30 (4–84)	−0.52 ± 0.94	64.30 ± 11.24 (37–91)	0.59 ± 0.69
PASAT3	40.83 ± 15.44 (0–60)	−0.40 ± 1.14	52.45 ± 7.26 (24–60)	0.45 ± 0.54
PASAT2	30.18 ± 14.02 (0–60)	−0.41 ± 1.06	41.55 ± 8.94 (18–60)	0.46 ± 0.68
WLG	21.95 ± 7.21 (2–37)	−0.40 ± 1.02	27.99 ± 5.29 (12–40)	0.45 ± 0.75

For each test, data are expressed as mean ± standard deviation scores (ranges). MS patient scores were significantly different from healthy control scores for all tests ($p < 0.0001$).

1.00–7.24), and 13.54 ± 9.32 (range, 0–45), respectively. Corresponding scores for healthy controls were 12.03 ± 5.55 (range, 0–27), 3.40 ± 0.89 (range, 1.00–5.41), and 9.47 ± 6.59 (range, 0–27). For all 3 instruments, MS patients scored significantly higher compared to healthy controls ($p = 0.0007$, $p < 0.0001$, and $p < 0.0001$, respectively), suggesting the presence of more apathy, more fatigue, and more depression in patients. In MS patients, AS, FQ, and BDI-II scores were not associated with disease duration. On the other hand, positive correlations were noted between scores on the AS, FQ, or BDI-II and the EDSS in MS patients ($\gamma = 0.17$, $p < 0.05$; $\gamma = 0.15$, $p < 0.05$; and $\gamma = 0.20$, $p < 0.01$; respectively).

Relationship between cognitive performance and measures of apathy, fatigue, and depression

Next we evaluated whether apathy, fatigue, and depression were correlated with the BRB-N. Table 4 shows that in healthy controls, AS and FQ scores were not correlated with BRB-N scores. However, SRT-LTS, SRT-CLTR, SDMT, PASAT3, and PASAT2 scores were correlated with BDI-II score. On the other hand, in patients with MS, most test scores of the BRB-N were correlated with the scores on the AS and BDI-II. However, FQ score was not correlated with any of the BRB-N tests except WLG.

Effect of patient, apathy, fatigue, and depression in the BRB-N

To examine how much each of the patient, apathy, fatigue, and depression factors affect the BRB-N score, multiple regression analysis was conducted with these 4 factors as explanatory variables for each BRB-N test. In this analysis, "patient" was defined as 1 and "healthy control" as 0. It was found that only "patient" had a significant effect in all tests, indicating that differences in BRB-N scores between MS patients and healthy controls remained significant even after controlling for the effects of apathy, fatigue, and depression (Table 5).

Table 2 Correlation between age at examination or duration of education and the BRB-N

	Age at examination				Duration of education			
	MS patients		Healthy controls		MS patients		Healthy controls	
BRB-N	γ	*p* value	γ	*p* value	γ	*p* value	γ	*p* value
SRT-LTS	−0.23	0.0017	−0.53	<0.0001	0.21	0.0045	0.22	0.0055
SRT-CLTR	−0.25	0.0006	−0.55	<0.0001	0.17	0.0214	0.19	0.0155
SRT-D	−0.16	0.0318	−0.53	<0.0001	0.17	0.0199	0.21	0.0084
SPART	−0.22	0.0023	−0.32	<0.0001	0.11	n.s.	0.07	n.s.
SPART-D	−0.24	0.0009	−0.25	0.0011	0.07	n.s.	0.06	n.s.
SDMT	−0.24	0.0012	−0.44	<0.0001	0.12	n.s.	0.23	0.0027
PASAT3	−0.13	n.s.	−0.25	0.0014	0.13	n.s.	0.15	n.s.
PASAT2	−0.10	n.s.	−0.31	<0.0001	0.12	n.s.	0.19	0.0150
WLG	−0.10	n.s.	−0.11	n.s.	0.04	n.s.	−0.09	n.s.

n.s.: not significant ($p > 0.05$).

Table 3 Correlation between disease duration or EDSS score and BRB-N test scores in patients with MS

	Disease duration		EDSS	
BRB-N	γ	*p* value	γ	*p* value
SRT-LTS	−0.16	0.0271	−0.37	<0.0001
SRT-CLTR	−0.19	0.0093	−0.34	<0.0001
SRT-D	−0.18	0.0120	−0.37	<0.0001
SPART	−0.22	0.0023	−0.25	0.0005
SPART-D	−0.24	0.0010	−0.28	0.0002
SDMT	−0.18	0.0133	−0.49	<0.0001
PASAT3	−0.24	0.0012	−0.42	<0.0001
PASAT2	−0.18	0.0141	−0.40	<0.0001
WLG	−0.09	n.s.	−0.33	<0.0001

n.s.: not significant ($p > 0.05$).

Discussion

Some degree of cognitive impairment is found in at least half of all patients with MS, and cognitive impairment typically consists of domain-specific deficits rather than global cognitive decline [9,22]. Cognitive impairment may be affected by environmental and educational factors, and there have been no large population studies on cognitive function in Japanese patients with MS. The BRB-N is now widely accepted for use in clinical studies [23] as well as in clinical practice [7]. Furthermore, studies in several populations using the BRB-N have revealed that the battery is largely unaffected by language or cultural differences, thereby validating its use in different populations [6,7,24]. The values obtained from the healthy control group in our study were similar to those found in Dutch [6], Italian [24], and Spanish [7] populations, indicating that our Japanese version did not influence performance on the test.

PASAT is a complex task and its performance largely depends on information-processing speed and working memory, which are two important and separate cognitive processes involved in the execution of the test [25]. Although the PASAT involves a larger number of cognitive processes, the SDMT could provide a better index of information-processing speed, which seems to be more frequently impaired in patients with MS [25,26]. Further, SDMT is a good test to predict cognitive impairment in patients with MS, even in the early stages of the disease [27]. Our data demonstrate that cognitive function is impaired also in Japanese patients with MS, especially in terms of information-processing speed and attentional deficits, as shown by their SDMT and PASAT scores.

Previous studies demonstrated that physical disability evaluated by EDSS score was independently associated with cognitive impairment evaluated by the BRB-N [7,24,26]. We also demonstrated a correlation between physical disability and cognitive impairment in the present Japanese MS population. These data suggest that inhibition of relapses and improved prognosis with disease-modifying therapies will also benefit cognitive function.

Some previous studies suggested that cognitive performance does not seem to correlate significantly with disease duration [22,24]; however, longitudinal studies suggest that cognitively impaired patients experience ongoing cognitive decline [1,28]. The reason for these conflicting results remains unclear, although the proportion of patients with different MS subtypes (primary progressive, relapsing–remitting, and secondary progressive) or patient age may be important. Previous studies suggest that long-term treatment with IFNβ may protect against cognitive impairment in patients with MS [29,30]. In our study, there were not any significant differences in all 9 BRB-N tests between MS patients who received IFNβ and those who did not receive IFNβ, however, the durations of IFNβ treatment were various. It is difficult to conclude effects of IFNβ treatment on cognitive function

Table 4 Correlation between apathy (apathy scale), fatigue (fatigue questionnaire), and depression (BDI-II) and the BRB-N

	Apathy				Fatigue				Depression			
	MS patients		Healthy controls		MS patients		Healthy controls		MS patients		Healthy controls	
BRB-N	γ	*p* value	γ	*p* value	γ	*p* value	γ	*p* value	γ	*p* value	γ	*p* value
SRT-LTS	−0.23	0.0018	−0.04	n.s.	0.05	n.s.	0.02	n.s.	−0.18	0.0208	−0.18	0.0226
SRT-CLTR	−0.22	0.0031	−0.04	n.s.	0.04	n.s.	0.02	n.s.	−0.13	n.s.	−0.16	0.0370
SRT-D	−0.23	0.0014	0.00	n.s.	0.05	n.s.	0.01	n.s.	−0.14	n.s.	−0.11	n.s.
SPART	−0.27	0.0003	−0.00	n.s.	−0.02	n.s.	−0.09	n.s.	−0.18	0.0185	−0.02	n.s.
SPART-D	−0.33	<0.0001	−0.03	n.s.	−0.01	n.s.	−0.04	n.s.	−0.16	0.0446	−0.08	n.s.
SDMT	−0.28	0.0002	−0.07	n.s.	−0.03	n.s.	−0.01	n.s.	−0.28	0.0002	−0.29	0.0002
PASAT3	−0.22	0.0033	0.12	n.s.	−0.04	n.s.	−0.06	n.s.	−0.25	0.0013	−0.21	0.0083
PASAT2	−0.21	0.0047	0.01	n.s.	−0.04	n.s.	−0.14	n.s.	−0.23	0.0031	−0.29	0.0002
WLG	−0.23	0.0016	−0.07	n.s.	0.16	0.03	0.03	n.s.	−0.15	0.0458	−0.15	n.s.

n.s.: not significant ($p > 0.05$).

Table 5 Effect of patient, apathy, fatigue, and depression factors in the BRB-N tests

	SRT-LTS		SRT-CLTR		SRT-D		SPART		SPART-D	
Explanatory variable	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**
Patient	−0.3135	<0.0001	−0.3465	<0.0001	−0.3189	<0.0001	−0.3591	<0.0001	−0.2706	<0.0001
Apathy	−0.1249	0.0314	−0.1154	0.0470	−0.1379	0.0191	−0.1107	n.s.	−0.1807	0.0025
Fatigue	0.1713	0.0035	0.1362	0.0199	0.1245	0.0352	0.0326	n.s.	0.060	n.s.
Depression	−0.1916	0.0028	−0.1405	0.0281	−0.1190	n.s.	−0.0803	n.s.	−0.0667	n.s.
Adjusted R-squared	0.1684		0.1668		0.1485		0.1659		0.1243	
	SDMT		**PASAT3**		**PASAT2**		**WLG**			
Explanatory variable	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**	**Standard estimate (β)**	***p* value**		
Patient	−0.5251	<0.0001	−0.3823	<0.0001	−0.3858	<0.0001	−0.4319	<0.0001		
Apathy	−0.0764	n.s.	−0.0176	n.s.	−0.0108	n.s.	−0.1511	0.0058		
Fatigue	0.1340	0.0074	0.0842	n.s.	0.0673	n.s.	0.2171	<0.0001		
Depression	−0.2664	<0.0001	−0.2471	<0.0001	−0.2516	<0.0001	−0.1708	0.0046		
Adjusted R-squared	0.3933		0.2221		0.2301		0.2647			

n.s.: not significant ($p > 0.05$).

in MS from our study, and further studies are needed about effects of DMDs on cognitive function.

In the present study, we aimed to evaluate correlations between cognitive impairment and the three factors of apathy, fatigue, and depression in MS patients. Our results demonstrate that MS patients had more apathy, more fatigue, and more depression compared with healthy controls, and decreased cognitive function was correlated with apathy and depression in Japanese patients with MS. Despite the association between cognitive variables and depression/apathy, cognitive function was impaired beyond the effect of depression and apathy. No associations between disease duration and scores on the AS, FQ, or BDI-II were found although positive correlations between EDSS and all 3 scores were found in MS patients. Other studies also demonstrated no significant longitudinal change in the Fatigue Severity Scale across a 2- to 3-year interval in patients with MS [31], and fatigue was not correlated with disease duration [32]. Together these previous and the present findings suggest that disease duration may have little association with subjective fatigue.

Apathy is one of the major neuropsychiatric symptoms in patients with MS [33]. Figved et al. reported that apathy was significantly associated with intrusions in patients with MS [34], although few studies have explored the relationship between cognitive impairment and apathy. We demonstrated impaired apathy in Japanese patients with MS compared to healthy controls, and a negative correlation was found between apathy and cognitive function. Future studies of cognitive function should also focus on apathy.

Fatigue is a common symptom of MS, and patients with MS often report a correlation between self-reported fatigue and their perception of poor performance on cognitive tests [35]. However, no relationship has been reported between fatigue and cognitive impairment [33,36]. Our results support these findings, and subjective fatigue may not be strongly associated with cognitive impairment in MS patients. However, differences exist between subjective and objective cognitive fatigue [37]. Furthermore, fatigue could lead to unemployment in MS patients and thus a reduction in quality of life [38], and it is therefore important to investigate cognitive function and subjective fatigue using different approaches.

The prevalence of major depression in patients with MS is relatively high [39] and this may affect cognitive function. Indeed, it was reported that depression influences cognitive performance [40], although in another study depression it was not found to correlate with cognitive function [41]. Despite previous inconsistent findings regarding the association between depression and cognitive function, our results demonstrated that depression was correlated with the individual tests of the BRB-N. BDI-II is an instrument to measure the severity of depression, not to diagnose major depressive disorder. Our data of BDI-II demonstrated MS patients scored significantly higher compared to healthy controls, and suggested that MS patients may suffer from sub-depressive conditions.

Conclusions

Cognitive function, in particular information-processing speed, was impaired and decreased cognitive function was correlated with apathy and depression in Japanese patients with MS. However, subjective fatigue was not associated with cognitive dysfunction. Both fatigue and cognition affect quality of life for patients with MS, and

we may need to consider therapeutic intervention to improve fatigue and cognition using different approaches.

Competing interests

MN has received funding for travel and/or speaker honoraria from Biogen Idec, Bayer Schering Pharma, and Novartis Pharma, and has served on the scientific advisory boards for Biogen Idec. T. Kohriyama has received speaker honoraria from Biogen Idec, Bayer Yakuhin Ltd., and Novartis Pharma. IK has received funding for travel and/or speaker honoraria from Novartis Pharma, Biogen Idec, and Bayer Schering Pharma. YS has received honoraria for speaking from Bayer Yakuhin Ltd., and has received personal compensation for consulting services from Biogen Idec, Teijin Pharma and Novartis Pharma. HF has received funding for travel and/or speaker honoraria from Biogen Idec, Daiichi Sankyo Inc., Dainippon Sumitomo Pharma and Novartis Pharma. IN has served on the scientific advisory boards for Biogen Idec, Novartis Pharma; received funding for trips and speaks from Bayer Yakuhin Ltd., Biogen Idec, Tanabe Mitsubishi Pharma, Novartis Pharma, and received grant support from Mitsubishi Chemical Medience Corporation. S. Kusunoki has received speaker honoraria from Teijin, Nihon Pharmaceutical, Benesis, Japan Blood Products Organization, Novartis Pharma, Asahi Kasei, and Sanofi Aventis. KN has received funding for travel and/or speaker honoraria from Biogen Idec, Bayer Yakuhin Ltd., Mitsubishi Tanabe Pharma, Nihon Pharmaceutical Co., Ltd., Teijin Pharma Ltd., and Novartis Pharma. TY has served on scientific advisory boards for Biogen Idec and Chugai Pharmaceutical Co., Ltd.; has received research support from Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., Teva Pharmaceutical K.K., Mitsubishi Tanabe Pharma, and Asahi Kasei Kuraray Medical CO., Ltd; has received speaker honoraria from Novartis Pharma, Nihon Pharmaceutical Co., Ltd., Santen Pharmaceutical Co., Ltd., Abbot Japan Co., Ltd., Eisai Co., Ltd., Biogen Idec, Dainippon Sumitomo Pharma Co., Ltd., Mitsubishi Tanabe Pharma, Bayer Holding Ltd., and Astellas Pharma Inc. JK is a consultant for Biogen Idec, and has received honoraria from Bayer Healthcare and funding for a trip from Bayer Healthcare and Biogen Idec. M. Matsui is part of a scientific advisory board for Biogen Idec, and has received speaker honoraria from Bayer Healthcare, Biogen Idec, and Tanabe Mitsubishi Pharma. YM has received speaker honoraria and research material from Novartis Pharma. S. Kikuchi has received speaker honoraria from Novartis Pharma, Boehringer Ingelheim, Kyowa Hakko Kirin, Dainippon Sumitomo Pharma, and FP Pharmaceutical Corporation, and serves on the scientific advisory board for Novartis Pharma. NM, M. Mori, TO, KM, K. Yoshida, T. Kanda, FY, SN, and K. Yokoyama declare that they have no competing interests.

Authors' contributions

MN was responsible for study design, data collection, and manuscript preparation. NM was responsible for statistical analysis and manuscript preparation. IK and KM were responsible for study design, data collection, and manuscript review. S. Kusunoki and S. Kikuchi were responsible for study design and manuscript review. T. Kohriyama, M. Mori, TO, YS, HF, IN, K. Yoshida, T. Kanda, KN, TY, FY, JK, SN, K. Yokoyama, M. Matsui, and YM were responsible for data collection at their respective institutions and manuscript review. All authors read and approved the final manuscript.

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Author details

[1]Department of Clinical Research, Hokkaido Medical Center, Yamanote 5jo 7chome, Nishi-ku, Sapporo 063-0005, Japan. [2]School of Management, Kochi University of Technology, Kochi, Japan. [3]Department of Neurology, Hiroshima City Hospital, Hiroshima, Japan. [4]Department of Neurology, Graduate School of Medicine, Chiba University, Chiba, Japan. [5]Department of Neurology, Tokyo Women's Medical University Yachiyo Medical Center, Chiba, Japan. [6]Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan. [7]Department of Neurology, Tokyo Women's Medical University School of Medicine, Tokyo, Japan. [8]Department of Neurology, Iwate Medical School, Morioka, Japan. [9]Department of Neurology, Saitama Medical Center, Saitama Medical University, Saitama, Japan. [10]Department of Neurology, Tohoku University School of Medicine, Sendai, Japan. [11]Department of Neurology, Kinki University School of Medicine, Osaka, Japan. [12]Department of Neurology, Asahikawa Red Cross Hospital, Asahikawa, Japan. [13]Department of Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan. [14]Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan. [15]Department of Neurology, Tokai University School of Medicine, Kanagawa, Japan. [16]Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. [17]Department of Clinical Research, Nagasaki Kawatana Medical Center, Nagasaki, Japan. [18]Department of Neurology, Juntendo University School of Medicine, Tokyo, Japan. [19]Department of Neurology, Kanazawa Medical University, Ishikawa, Japan. [20]Department of Neurology, Hokkaido Medical Center, Sapporo, Japan.

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OX40 ligand regulates splenic CD8$^-$ dendritic cell-induced Th2 responses *in vivo*

Fumitaka Kamachi [a], Norihiro Harada [a,b], Yoshihiko Usui [a,c], Tamami Sakanishi [d], Naoto Ishii [e], Ko Okumura [a], Sachiko Miyake [a], Hisaya Akiba [a,*]

[a] Department of Immunology, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[b] Department of Respiratory Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[c] Department of Ophthalmology, Tokyo Medical University, 6-7-1 Nishi-Shinjuku-ku, Tokyo 160-0023, Japan
[d] Division of Cell Biology, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[e] Department of Microbiology and Immunology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan

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ABSTRACT

In mice, splenic conventional dendritic cells (cDCs) can be separated, based on their expression of CD8α into CD8$^-$ and CD8$^+$ cDCs. Although previous experiments demonstrated that injection of antigen (Ag)-pulsed CD8$^-$ cDCs into mice induced CD4 T cell differentiation toward Th2 cells, the mechanism involved is unclear. In the current study, we investigated whether OX40 ligand (OX40L) on CD8$^-$ cDCs contributes to the induction of Th2 responses by Ag-pulsed CD8$^-$ cDCs *in vivo*, because OX40–OX40L interactions may play a preferential role in Th2 cell development. When unseparated Ag-pulsed OX40L-deficient cDCs were injected into syngeneic BALB/c mice, Th2 cytokine (IL-4, IL-5, and IL-10) production in lymph node cells was significantly reduced. Splenic cDCs were separated to CD8$^-$ and CD8$^+$ cDCs. OX40L expression was not observed on freshly isolated CD8$^-$ cDCs, but was induced by anti-CD40 mAb stimulation for 24 h. Administration of neutralizing anti-OX40L mAb significantly inhibited IL-4, IL-5, and IL-10 production induced by Ag-pulsed CD8$^-$ cDC injection. Moreover, administration of anti-OX40L mAb with Ag-pulsed CD8$^-$ cDCs during a secondary response also significantly inhibited Th2 cytokine production. Thus, OX40L on CD8$^-$ cDCs physiologically contributes to the development of Th2 cells and secondary Th2 responses induced by Ag-pulsed CD8$^-$ cDCs *in vivo*.

1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells critical for the induction of adaptive immune responses. Conventional DCs (cDCs) are specialized for antigen processing and presentation to T cells and can be subdivided by their surface expression of CD8α and CD4 as CD8$^-$CD4$^+$, CD8$^-$CD4$^-$, and CD8$^+$CD4$^-$ cDCs in the spleen [1–4]. Both CD8$^-$CD4$^+$ and CD8$^-$CD4$^-$ cDCs appear functionally similar and are referred to as CD8$^-$ cDCs [2,3]. In contrast, the physiologic functions of both CD8$^-$ cDCs and CD8$^+$ cDCs markedly differ. *In vivo* experiments demonstrated that injection of antigen-pulsed CD8$^-$ cDCs induced CD4 T cell differentiation toward Th2 responses (high levels of IL-4, IL-5, and IL-10) whereas antigen-pulsed CD8$^+$ cDCs induced Th1 responses (high levels of IFN-γ [5]. The ability of CD8$^+$ cDCs to induce Th1 differentiation is explained by their ability to produce IL-12 efficiently [6,7]. However, the mechanisms of Th2 responses induced by CD8$^-$ cDCs are not understood.

CD4 T cell differentiation might be regulated by cytokines and various costimulatory molecules expressed on CD4 T cells, and their cognate ligands expressed on DCs such as OX40 (CD134) costimulatory molecule, a member of the TNF receptor superfamily, and its ligand, OX40L (CD252) [8,9]. OX40 is preferentially expressed on activated CD4 T cells and OX40L is mainly expressed on antigen-presenting cells, including activated DCs, B cells, and macrophages. Recent studies emphasized the role of OX40L on DCs for Th2 polarization. In humans, schistosomal egg antigen induced monocyte-derived DCs to express OX40L, which contributed to the induction of Th2 responses [10]. IL-3-treated plasmacytoid DCs expressed OX40L and induced Th2 responses by promoting CD4 T cells to secrete IL-4, IL-5, and IL-13. Blockade of OX40L significantly inhibited this ability of IL-3-treated plasmacytoid DCs [11]. Moreover, OX40L expressed on thymic stromal lymphopoietin (TSLP)-activated DCs induced naïve CD4 T cells to differentiate into TNF-α$^+$ IL-10$^-$ inflammatory Th2 cells [12]. In mice, OX40L expression on bone marrow-derived DCs (BMDCs) is upregulated downstream of CD40 signaling and is critical for optimal Th2 priming *in vivo* [13]. In contrast to these studies, the use of agonistic anti-OX40 mAb revealed OX40-mediated costimulation enhanced the

* Corresponding author. Fax: +81 3 3813 0421.
E-mail address: hisaya@juntendo.ac.jp (H. Akiba).

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development of Th1 responses induced by splenic $CD8^-$ cDCs *in vivo* [14]. Thus, the function of OX40L on splenic $CD8^-$ cDCs is still controversial. In this study, we examined the physiological contribution of OX40–OX40L interactions on $CD8^-$ cDCs-induced Th2 responses by using blocking anti-OX40L mAb.

2. Materials and methods

2.1. Animals

Female BALB/c mice were purchased from Charles River Laboratories (Kanagawa, Japan). OX40L-deficient mice were generated as previously described [15] and backcrossed for seven generations with BALB/c mice purchased from Oriental Yeast Co. (Tokyo, Japan). All mice were 6–8 week old at the start of experiments and kept under specific pathogen-free conditions during the experiments. All animal experiments were approved by Juntendo University Animal Experimental Ethics Committee.

2.2. Antibodies and reagents

An anti-mouse OX40L (RM134L) mAb was previously generated in our laboratory [16]. Control rat IgG was purchased from Sigma–Aldrich (St Louis, MO, USA). Purified anti-CD40 (HM40-3), allophycocyanin (APC)-conjugated anti-CD8α (53-6.7), and rat IgG isotype control were purchased from eBioscience (San Diego, CA, USA). Purified anti-CD16/32 (2.4G2) and FITC-conjugated anti-CD11c (HL3), recombinant mouse GM-CSF, IL-4, and IFN-γ were purchased from BD Biosciences (San Jose, CA, USA).

2.3. Preparation and stimulation of splenic DCs

To isolate splenic DCs, spleens from BALB/c or OX40L-deficient mice were digested with 400 U/ml of collagenase (Wako Biochemicals, Tokyo, Japan), further dissociated in Ca^{2+}-free medium in the presence of 5 mM EDTA, and separated into low- and high-density fractions by Optiprep-gradient (Axis-Shield, Oslo, Norway) as described previously [17]. Low-density cells were pulsed overnight with 50 μg/ml of keyhole limpet hemocyanin (KLH) in culture medium supplemented with 20 ng/ml of GM-CSF as described previously [5]. After overnight culture, splenic $CD11c^+$ DCs were isolated by incubation with anti-CD11c-coupled magnetic beads and positive selection by autoMACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD11c^+$ DCs were further separated according to CD8α expression by FACS sorting. $CD11c^+$ cells were incubated with FITC-conjugated anti-CD11c and APC-conjugated anti-CD8α mAbs, and two populations ($CD8^+CD11c^+$ DCs and $CD8^-CD11c^+$ DCs) were sorted by FACSVantage (BD Biosciences). To examine OX40L expression, separated DC populations were incubated with anti-CD40 mAb (10 μg/ml) with IL-4 (20 ng/ml) or IFN-γ (20 ng/ml) in the presence or absence of GM-CSF (20 ng/ml) at 37 °C for 24 h.

2.4. Flow cytometric analysis

Cells were pre-incubated with unlabeled anti-CD16/32 mAb to avoid non-specific binding of Abs to FcγR, incubated with FITC- or APC-labeled mAbs, or biotinylated mAb followed by PE-labeled streptavidin. Stained cells (live cells gated by forward and side scatter profiles and propidium iodide exclusion) were analyzed by FACSCalibur (BD Biosciences), and data were processed by CellQuest (BD Biosciences).

2.5. Immunization protocol

KLH-pulsed splenic cDCs were washed in PBS and immunized (3×10^5 cells) into the hind footpad of BALB/c mice. Some groups of mice ($n = 5$–6) were administered 400 μg of anti-OX40L mAb or rat IgG intraperitoneally (i.p.) at days 0, 1, and 3, or daily from days 0 to 3 and days 14–17. Popliteal lymph node (LN) cells were harvested 5 days after primary or secondary immunizations.

2.6. T cell stimulation in vitro

LN cells were isolated and cultured in RPMI1640 medium (containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-mercaptoethanol) at a density of 6×10^5 cells/well in the presence of indicated doses of KLH. To assess proliferative responses, cultures were pulsed with tritiated thymidine ($[^3H]$TdR; 0.5 μCi/well; PerkinElmer, Winter Street Waltham, MA, USA) for the last 6 h of a 48 h or 72 h culture and harvested on a Micro 96 Harvester (Molecular Devices, Sunnyvale, CA, USA). Incorporated radioactivity was measured using a microplate beta counter (Micro β Plus; PerkinElmer). To determine cytokine production, cell-free supernatants were collected at 48 h or 72 h and assayed for IL-2, IL-4, IL5, IL-10, and IFN-γ by ELISA using Ready-SET-Go! kits (eBioscience) according to the manufacturer's instructions.

2.7. Statistical analysis

Statistical analyses were performed by unpaired Student *t*-test or Tukey's multiple comparison test. Results are expressed as mean ± SEM. Values of $P < 0.05$ were considered significant.

3. Results

3.1. OX40L is required for optimal Th2 responses induced by splenic cDCs in vivo

Because a previous report demonstrated KLH-pulsed $CD8^-$ and $CD8^+$ cDCs differentially regulated Th cell development, we followed the same protocol using KLH as an antigen. To clarify the contribution of splenic cDC OX40L on CD4 T cell differentiation, we examined CD4 T cell responses induced by splenic $OX40L^{-/-}$ cDCs. cDCs were purified from spleens of OX40L-deficient or wild-type BALB/c mice without treatment, pulsed with KLH during overnight culture with GM-CSF, to isolate $CD11c^{high}$ $B220^-$ cells (cDC population). $OX40L^{-/-}$ cDCs or WT cDCs (3×10^5) were injected into hind footpads of syngeneic BALB/c mice. LNs were prepared on day 5 and proliferative responses and cytokine production against various doses of KLH were assessed. KLH-specific proliferative responses and IL-2 production were reduced in LN cells from $OX40L^{-/-}$ cDCs-injected mice compared with WT cDCs-injected mice (Fig. 1). Th2 cytokine production (IL-4, IL-5, and IL-10) was also significantly reduced in $OX40L^{-/-}$ cDCs-injected mice compared with WT cDCs-injected mice. In contrast, Th1 type cytokine IFN-γ production was non-significantly increased in $OX40L^{-/-}$ cDCs-injected mice compared with WT cDCs-injected mice.

Similar results were obtained when KLH-pulsed $OX40L^{-/-}$ bone marrow-derived DCs (BMDCs) were injected into hind footpads of BALB/c mice (Supplemental Fig. S1). KLH-specific proliferative responses and IL-2 production were reduced in LN cells from $OX40L^{-/-}$ BMDCs-injected mice compared with WT BMDCs-injected mice. Th2 cytokine production (IL-4, IL-5, and IL-10) was significantly reduced in $OX40L^{-/-}$ BMDCs-injected mice, whereas IFN-γ production was similar between $OX40L^{-/-}$ BMDCs-injected

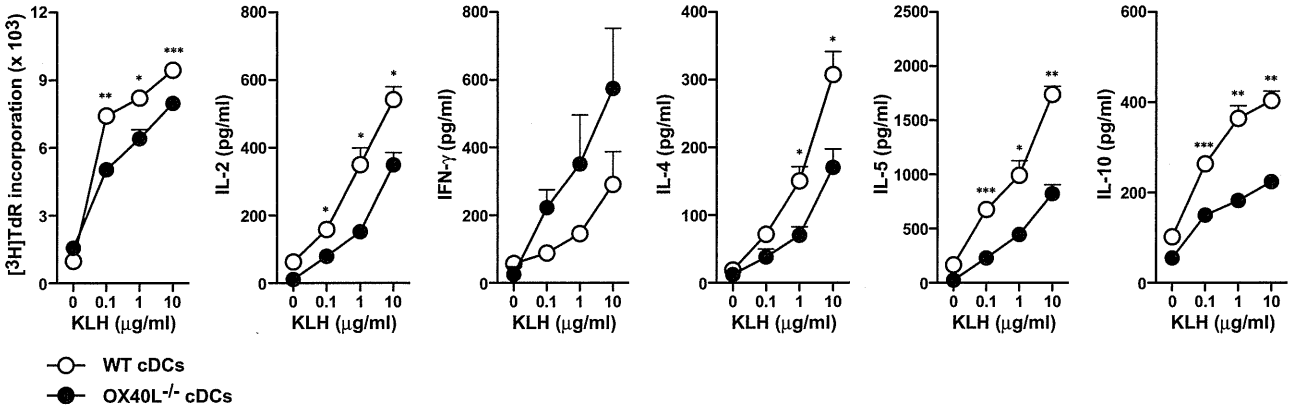

Fig. 1. OX40L is required for optimal Th2 responses by splenic cDCs *in vivo*. BALB/c mouse hind footpads were injected with KLH-pulsed cDCs isolated from the spleen of wild-type BALB/c or OX40L$^{-/-}$ BALB/c mice. LN cells were harvested at day 5 and cultured with indicated doses of KLH. To estimate proliferation, 0.5 μCi ^{3}H-thymidine ([^{3}H]TdR) was added during the last 6 h of a 48 h culture. Production of IFN-γ, IL-2, IL-4, IL-5, and IL-10 in culture supernatants at 48 h was determined by ELISA. Results are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Similar results were obtained in three independent experiments.

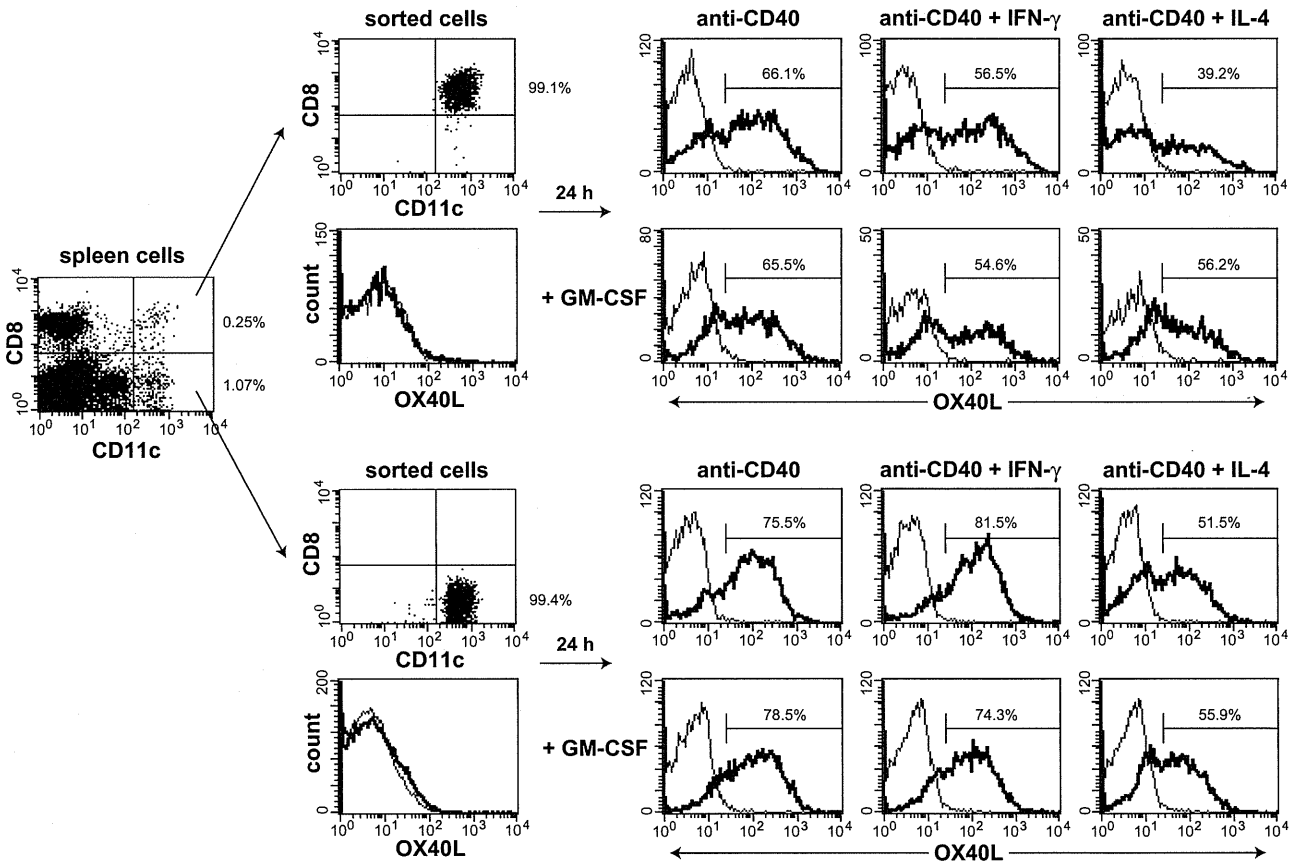

Fig. 2. Expression of OX40L on activated CD8^{-} and CD8^{+} cDCs. Spleen cells were isolated from BALB/c mice and stained with FITC-labeled anti-CD11c, APC-labeled anti-CD8α, and biotinylated anti-OX40L or control IgG followed by PE-labeled streptavidin. CD8^{-}CD11c^{high} and CD8^{+}CD11c^{high} cDCs were isolated from spleens by FACS sorting. Isolated CD8^{-}CD11c^{high} and CD8^{+}CD11c^{high} cDCs were stimulated with anti-CD40 mAb in the presence or absence of GM-CSF, IFN-γ, and IL-4. Cells were harvested at 24 h and stained with anti-OX40L mAb or control rat IgG. Thick lines indicate staining with anti-OX40L mAb and thin lines indicate background staining with control IgG. Data are representative of three experiments.

and WT BMDCs-injected mice. In addition, administration of neutralizing anti-OX40L mAb to WT BMDCs-injected mice significantly reduced Th2 cytokine production similar to OX40L$^{-/-}$ BMDCs-injected mice. Th2 cytokine reduction was also observed in KLH-pulsed WT BMDCs injected with anti-OX40L mAb into IFN-γ-deficient mice (Supplemental Fig. S2). These results indicated a critical role of OX40L in splenic cDCs- and BMDCs-induced Th2 responses *in vivo*. The inhibition of Th2 responses by anti-OX40L treatment was not necessarily a result of a shift to Th1 responses.

3.2. Expression of OX40L on splenic cDCs

The expression of OX40L on two major subsets of splenic cDCs was assessed by flow cytometry. Splenic cDCs were separated

based on CD8α and CD11c expression, into $CD8^{-}CD11c^{high}$ cDCs ($CD8^{-}$ cDCs) and $CD8^{+}CD11c^{high}$ cDCs ($CD8^{+}$ cDCs), and stimulated with agonistic anti-CD40 with or without cytokines (GM-CSF, IFN-γ, or IL-4) for 24 h (Fig. 2). While OX40L expression was not observed on freshly isolated $CD8^{-}$ or $CD8^{+}$ cDCs, it was induced by anti-CD40 mAb stimulation. Addition of IL-4 reduced OX40L expression on anti-CD40-stimulated $CD8^{-}$ and $CD8^{+}$ cDCs, whereas OX40L expression was not affected by the addition of GM-CSF or IFN-γ.

3.3. Effect of anti-OX40L mAb on the development of Th2 responses induced by KLH-pulsed $CD8^{-}$ cDCs in vivo

We next examined whether KLH-pulsed $CD8^{-}$ cDCs could induce Th2 responses compared with KLH-pulsed $CD8^{+}$ cDCs, and whether OX40L contributes to $CD8^{-}$ cDCs-induced Th2 responses. BALB/c mice were injected into the hind footpads with KLH-pulsed $CD8^{-}$ or $CD8^{+}$ cDCs, and treated with anti-OX40L mAb or control IgG at days 0, 1, and 3. LN cells were isolated at day 5 and KLH-specific proliferative responses and cytokine production were assessed. Consistent with previous reports, IL-4 production by LN cells from $CD8^{-}$ cDCs-injected mice was significantly higher than in $CD8^{+}$ cDCs-injected mice (Fig. 3). In contrast, IFN-γ production in $CD8^{+}$ cDCs-injected mice was non-significantly increased compared with the $CD8^{-}$ cDCs-injected mice. Proliferative responses and other Th2 cytokine production (IL-5 and IL-10) were similar between $CD8^{-}$ cDCs-injected and $CD8^{+}$ cDCs-injected mice. Anti-OX40L mAb administration strongly inhibited IL-4, IL-5, and IL-10 production induced by $CD8^{-}$ cDCs injection, while IFN-γ was slightly increased. Thus, OX40L has an important role in the development of Th2 responses induced by KLH-pulsed $CD8^{-}$ cDCs *in vivo*. Furthermore, administration of anti-OX40L mAb reduced IL-4 production induced by $CD8^{+}$ cDCs injection. Therefore, OX40L may also regulate IL-4 production induced by KLH-pulsed $CD8^{+}$ cDCs.

3.4. Effect of anti-OX40L mAb in secondary Th2 responses induced by KLH-pulsed $CD8^{-}$ cDCs in vivo

The OX40–OX40L pathway is crucial for recall responses when memory T cells are reactivated [18]. Therefore, we further examined the role of OX40L in secondary Th2 responses induced by KLH-pulsed $CD8^{-}$ cDCs *in vivo*. BALB/c mice were immunized first into the hind footpads with KLH-pulsed $CD8^{-}$ cDCs at day 0 and then under the same conditions with KLH-pulsed $CD8^{-}$ cDCs at day 14. Some groups of mice were treated with anti-OX40L mAb or control IgG daily from days 0 to 3 in the primary phase and days 14–17 in the secondary phase. LN cells were isolated at day 19 and the KLH-specific Th2 cytokine production was assessed. Anti-OX40L mAb administration during the primary phase only, reduced IL-4 and IL-5 production compared with control IgG (Fig. 4). In addition, anti-OX40L mAb administration in the secondary phase strongly inhibited IL-4, IL-5, and IL-10 production compared with control IgG. The inhibitory effect of anti-OX40 mAb

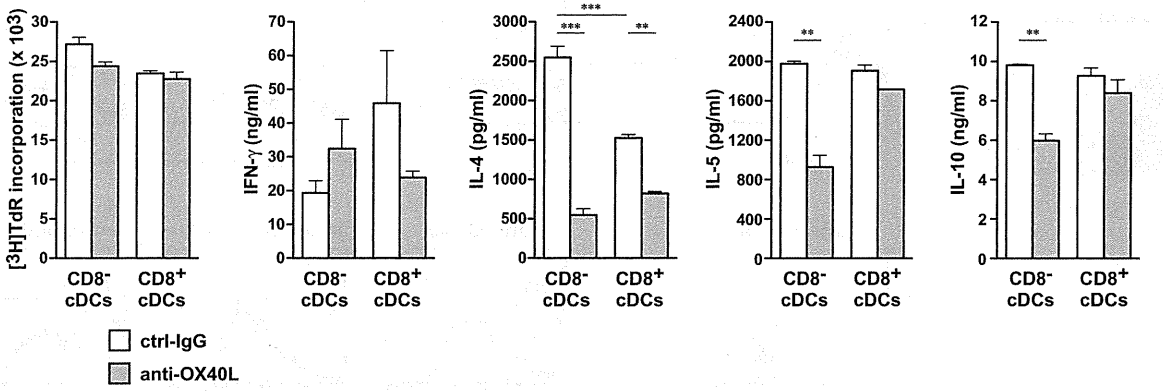

Fig. 3. Effect of anti-OX40L mAb on the development of Th2 responses induced by KLH-pulsed $CD8^{-}$ cDCs *in vivo*. BALB/c mouse hind footpads were injected with KLH-pulsed $CD8^{-}$ or $CD8^{+}$ cDCs. Mice were administered 400 μg of anti-OX40L mAb or control rat IgG (ctrl-IgG) i.p. at days 0, 1, and 3. LN cells were harvested at day 5 and cultured with 20 μg/ml of KLH. To estimate proliferation, 0.5 μCi [^{3}H]TdR was added during the last 6 h of a 72 h culture. Production of IFN-γ, IL-4, IL-5, and IL-10 in the culture supernatants at 72 h was determined by ELISA. Results are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Similar results were obtained in three independent experiments.

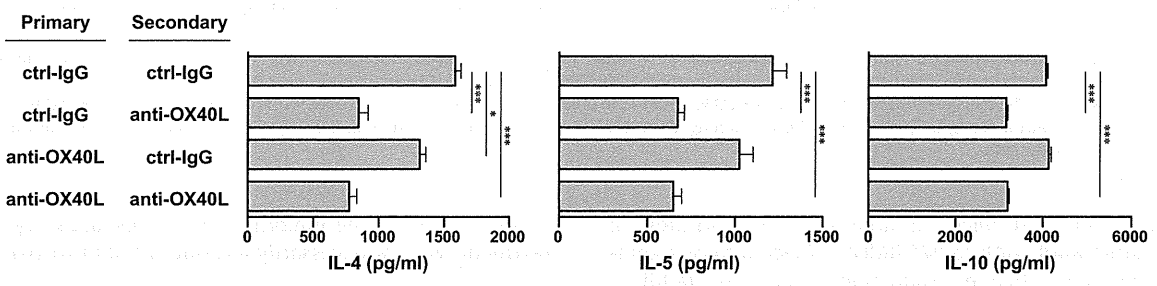

Fig. 4. Effect of anti-OX40L mAb on the development of memory Th2 responses induced by $CD8^{-}$ cDCs *in vivo*. BALB/c mice were immunized first with KLH-pulsed $CD8^{-}$ cDCs at day 0 and boosted with the same KLH-pulsed $CD8^{-}$ cDCs at day 14. Mice were administered 400 μg of anti-OX40L mAb or ctrl-IgG i.p. daily from days 0 to 3 and days 14–17. LN cells were harvested at day 19 and cultured with 10 μg/ml of KLH. To estimate proliferation, 0.5 μCi [^{3}H]TdR was added during the last 6 h of a 72 h culture. Production of IFN-γ, IL-4, IL-5, and IL-10 in culture supernatants at 72 h was determined by ELISA. Results are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Similar results were obtained in three independent experiments.

treatment in the secondary phase was comparable to mice treated with anti-OX40 mAb in both primary and secondary phases. Thus, OX40L might have an important role in both primary and secondary Th2 responses induced by KLH-pulsed $CD8^-$ cDCs *in vivo*.

4. Discussion

The current study investigated the physiological role of splenic $CD8^-$ cDC OX40L to regulate CD4 T cell Th2 differentiation *in vivo*. When antigen KLH-pulsed OX40L-deficient cDCs were injected into BALB/c mice, LN Th2 cytokine production (IL-4, IL-5, and IL-10) was significantly reduced. Splenic cDCs were separated into $CD8^-$ and $CD8^+$ cDCs. A previous study demonstrated that although injection of KLH-pulsed $CD8^-$ cDCs induced CD4 T cell differentiation toward Th2 responses, KLH-pulsed $CD8^+$ cDCs promoted Th1 responses [5]. Consistently, our results indicated that $CD8^-$ cDCs markedly induced IL-4 production and $CD8^+$ cDCs tended to induce IFN-γ production. Administration of neutralizing anti-OX40L mAb significantly inhibited IL-4, IL-5, and IL-10 production induced by KLH-pulsed $CD8^-$ cDCs. Moreover, treatment of anti-OX40L mAb with KLH-pulsed $CD8^-$ cDCs during a secondary response also significantly inhibited Th2 cytokine production. Thus, OX40L contributes to both the development of Th2 cells and secondary Th2 responses induced by KLH-pulsed $CD8^-$ cDCs *in vivo*. However, these findings are inconsistent with a previous report where administration of anti-OX40 mAb enhanced the development of Th1 cells secreting high levels of IFN-γ, but no IL-4 and IL-5, induced by KLH-pulsed $CD8^-$ cDCs *in vivo* [14]. The reason for this discrepancy is not clear, but it may be attributable to differences in experimental conditions. The previous study isolated splenic cDCs from mice treated with FMS-like tyrosine kinase 3 ligand (Flt3L) on 11 days, whereas mice were untreated in our study. Flt3 is a crucial factor in humans and mice to promote the development of cDCs *in vivo* and *in vitro*. However, a bias toward the generation of $CD8^+$ cDCs in the spleen was observed in mice treated with Flt3L [19,20]. The previous study also examined the effect of exogenous OX40 costimulation using agonistic anti-OX40 mAb, suggesting such an effect is not mediated by endogenous OX40–OX40L interactions between CD4 T cells and cDCs. Our results suggest that physiological OX40–OX40L interactions participate in CD4 T cell-$CD8^-$ cDCs interactions, and that OX40L on $CD8^-$ cDCs might contribute to the induction of Th2 responses *in vivo*.

In humans, TSLP-activated DCs can promote the differentiation of naïve CD4 T cells into a Th2 phenotype and the expansion of CD4 Th2 memory cells in an unique manner dependent on OX40L in the absence of IL-12 [12]. TSLP, an IL-7-like cytokine, is produced mainly by damaged epithelial cells and is a key molecule that links epithelial cells and DCs at the interface of allergic inflammation by participating in the programming of DC-mediated Th2 polarization [21–24]. TSLP activates STAT1, STAT3, STAT4, STAT5, and STAT6, whereas the contributions of individual STAT proteins to the activation of DCs is unclear [25]. Most recently, a mouse study demonstrated that DC-specific deletion of STAT5 was critical for TSLP-mediated Th2 differentiation, but not Th1 differentiation [26]. Loss of STAT5 in DCs affected upregulation of OX40L expression in response to TSLP. However, DC subsets in $Stat5^{-/-}$ chimeric mouse spleens had a higher proportion of $CD8^+$ cDCs and a reduced frequency of $CD4^+$ $CD8^-$ cDCs compared with $Stat5^{+/+}$ chimeras, suggesting STAT5 signaling regulates a balanced production of these splenic DC subsets *in vivo* [27]. Thus, STAT5 may be required for OX40L-dependent Th2 cell differentiation induced by KLH-pulsed $CD8^-$ cDCs. To confirm this, further studies are required using STAT5-specific deleted $CD8^-$ cDCs. In this study, we demonstrated that KLH-pulsed $OX40L^{-/-}$ BMDCs injected into hind footpads of BALB/c mice significantly reduced Th2 cytokine production (IL-4, IL-5, and IL-10) in LN cells compared with WT BMDCs-injected mice. Consistent with these observations, it was reported that OX40L expression by GM-CSF-induced BMDCs is required for optimal induction of primary and memory Th2 responses *in vivo* [13]. GM-CSF can activate STAT5, and GM-CSF-activated STAT5 inhibits the transcription of *Irf8* [27], which encodes interferon regulatory factor 8 (IRF8). IRF8 is required for IL-12 production [25], an essential cytokine required for the induction of Th1 responses [28]. Therefore, OX40L-dependent Th2 responses induced by KLH-pulsed $CD8^-$ cDCs might depend on the absence of IL-12, as IL-12 has a dominant effect over OX40L in Th cell differentiation [12]. Indeed, we observed that $CD8^+$ cDCs produced high amounts of IL-12p40 after stimulation with agonistic anti-CD40 mAb, whereas IL12p40 production on $CD8^-$ cDCs was markedly lower (unpublished observation). Taken together, these findings suggest that the development of Th2 responses by KLH-pulsed $CD8^-$ cDCs requires two conditions: the expression of OX40L and the absence of IL-12.

However, whether OX40 signaling on CD4 T cells directly induces Th2 differentiation is still unclear. It is well known that OX40 can bind to TNF receptor-associated factor (TRAF) 2, TRAF3, and TRAF5. However, these molecules also can bind to other TNF receptor family molecules. On a transcriptional basis, it was determined that OX40L expressed by TSLP-DCs induced the expression of GATA-3 in CD4 T cells, supporting their critical role in Th2 polarization [12]. Another study indicated that OX40 enhanced TCR-induced calcium influx, leading to the enhanced nuclear accumulation of NFATc1 and NFATc2, that likely regulates the production of cytokines [29]. More studies are required to determine how OX40 signaling promotes Th2 differentiation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.060.

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Ⅴ．研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

執筆者氏名	論文題名	書籍全体の編集者名	書籍名	出版社名	出版地	ページ	出版年・月
久松理一 （日比紀文，金井隆典　監修）	外来で診る消化管疾患		Medical Science Digest	北隆館	国内	p51-53	2014年8月

学会誌・雑誌等における論文掲載に関する一覧表

掲載した論文(発表題目)	発表者氏名	学会誌・雑誌名	出版年・月	国内・外の別	巻(号)	頁
Risk Factors for Decreased Bone Mineral Density in Japanese Patients with Inflammatory Bowel Disease: A Cross-Sectional Study.	Wada Y, Hisamatsu T* Naganuma M, Matsuoka K, Okamoto S, Inoue N, Yajima T, Kouyama K, Iwao Y, Ogata H, Hibi T, Abe T, and Kanai T.	Clin Nutr	2015 Jan	国外	S0261-5614(15)00008-4	
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Gut microbiota and inflammatory bowel disease.	Matsuoka K, Kanai T.	Seminars in Immunopathol.	2014 Nov	国外	37(1)	47-55
Classical Th1 cells obtain colitogenicity by co-existence of ROR γ t-expressing T cells in experimental colitis.	Saigusa K, Hisamatsu T, Handa T, Sujino T, Mikami Y, Hayashi A, Mizuno S, Takeshita K, Sato T, Matsuoka K, Kanai T.	Inflamm Bowel Dis.	2014 Oct	国外	20(10)	1820-7
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Endoscopic and pathologic changes of the upper gastrointestinal tract in Crohn's disease.	Sakuraba A, Iwao Y, Matsuoka K, Naganuma M, Ogata H, Kanai T, Hibi T.	Biomed Res Int.	2014 Feb	国外	2014	610767
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Fecal Microbiota Transplantation for Gastrointestinal Diseases	Matsuoka K, Mizuno S, Hayashi A, Hisamatsu T, Naganuma M and Kanai T.	Review Keio Journal of Medicine	2014 Dec	国外	63(4)	69-74
Early intervention with adalimumab may contribute to favorable clinical efficacy in patients with Crohn's disease.	Miyoshi J, Hisamatsu T, Matsuoka K, Naganuma M, Maruyama Y, Yoneno K, Mori K, Kiyohara H, Nanki K, Okamoto S, Yajima T, Iwao Y, Ogata H, Hibi T, Kanai T.	Digestion.	2014 Oct	国外	90(2)	130-6
Magnetic resonance enterography of Crohn's disease.	Naganuma M, Hisamatsu T, Kanai T, Ogata H.	Review Expert Rev Gastroenterol Hepatol.	2014 Sep	国外	9(1)	37-45
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