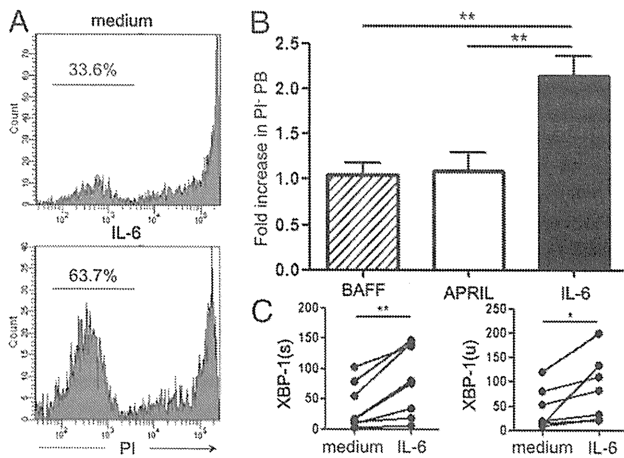

Fig. 4. Effect of exogenous IL-6 on PB. (*A*) IL-6 promotes the survival of PB. FACS-sorted PB were cultured in the presence or absence of recombinant IL-6 (1 ng/mL) for 2 d. PI staining of cultured PB showed that exogenous IL-6 increased the percentage of surviving cells (*Lower*) compared with cells cultured in the medium alone (*Upper*). Values shown are percentages of unstained cells. (*B*) Comparison of IL-6 with BAFF and APRIL. Here we show that only IL-6 could promote cell survival. Data are expressed as fold increase of % PI^- cells following the addition of each cytokine. At least four independent experiments were performed to obtain the results (** $P < 0.01$; Tukey's post hoc test). (*C*) Effect of IL-6 on XBP-1 expression. FACS-sorted PB were cultured with or without IL-6 for 24 h, and total RNA was extracted from the cells to quantify expression levels of XBP-1(u) and XBP-1(s) by qRT-PCR. Each line connects values obtained from seven independent experiments (* $P < 0.05$; ** $P < 0.01$; Wilcoxon signed-rank test).

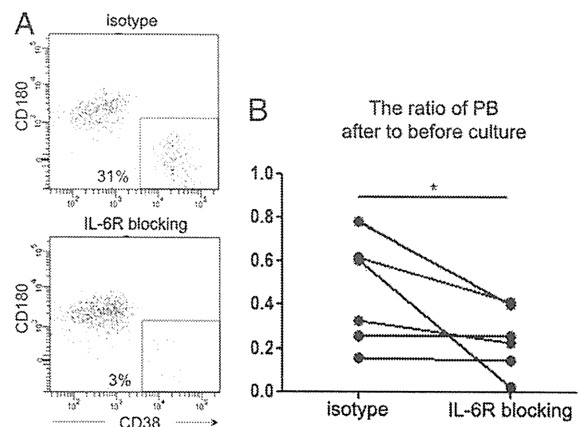

Fig. 5. IL-6R blockade selectively inhibits the survival of PB. (*A*) PBMC were cultured in a medium containing 20% autologous serum in the presence of IL-6R–blocking antibody or its isotype control mAb for 2 d. The cells were stained and analyzed as described in the experiment in Fig. 1*A*. Data represent the percentages of PB within $CD19^+CD27^+$ cells. A representative pair of six independent experiments is shown. (*B*) The percentage of PB within $CD19^+$ B cells (PB%) was determined for each pair of cultures either with anti–IL-6R mAb (IL-6R–blocking) or isotype control mAb (isotype) before and after starting the culture. Then, the PB survival ratio was calculated for each culture by dividing the PB% at the end of the culture by the PB% obtained before starting the culture. Lines connect the PB survival ratios of six independent experimental pairs to clarify that IL-6R blockade reduces PB survival (* $P < 0.05$; Wilcoxon signed-rank test).

dition of IL-6. These results suggest that IL-6 promoted the survival of PB and enhanced IgG secretion from PB, leading to an increased production of AQP4-Abs in NMO patients (Fig. 4*C*). In addition, we found that the frequency of PB tended to be increased when serum IL-6 levels were higher than the mean ± 2× SEM of those in HS [PB/PBMC (%) for IL-6 high group 0.62 ± 0.47 (%); PB/PBMC (%) for IL-6 low group 0.15 ± 0.05 (mean ± SD)]. These observations prompted us to address whether the blockade of IL-6R signaling could exhibit any influence on PB. We cultured PBMC derived from AQP4-Ab seropositive patients in the presence of 20% autologous serum and examined the effect of adding anti–IL-6R antibody by counting the number of surviving PB. We found that the frequency of PB among total B cells decreased significantly in the presence of anti–IL-6R mAb (Fig. 5 *A* and *B*). Among six patients examined, the PB reduction was remarkable in three patients, but was only marginal in the other patients. Notably, the former group of patients showed higher IL-6 levels in the serum (4.69, 6.47, and 25.5 pg/mL for each patient), compared with the latter (1.42, 1.43, and 2.91 pg/mL). The frequency of other B-cell subpopulations did not change with the addition of anti–IL-6R mAb. These results led us to postulate that in vivo administration of anti–IL-6R mAb may ameliorate NMO.

Discussion

A growing body of evidence suggests that AQP4-Abs play a pathogenic role in NMO (6, 7, 10–12). Here we report that a B-cell subpopulation bearing the $CD19^{int}CD27^{high}CD38^{high}CD180^-$ phenotype is responsible for the selective production of AQP4-Abs. The cells that we call PB are vulnerable to IL-6R blockade by anti–IL-6R mAb, leading us to propose anti–IL-6R mAb as a therapeutic option for NMO. Bennett et al. recently reported that plasma cells in CSF are a potential source of pathogenic AQP4-Abs (10). However, this study has not excluded a possible role of AQP4-Abs produced in the peripheral blood. It has been repeatedly shown that the passive transfer of pathogenic autoantibodies, including AQP4-Abs (10–12, 32), augments the formation of inflammatory lesions in EAE. Therefore, once T-cell–mediated inflammation takes place in the CNS, pathogenic autoantibodies produced outside the CNS are able to enter the CNS compartment. It is also notable that AQP4-Abs are more abundant in the peripheral blood of NMO patients than in their CSF (33). Taken together, we speculate that PB that are expanded in the peripheral blood during relapse may play a critical role in the pathogenesis of NMO by producing AQP4-Abs, although more work is necessary to explore whether PB can enter the CNS.

It is generally thought that circulating IgGs are mainly secreted by long-lived plasma cells residing in healthy bone marrow. It remains unclear how PB secreting AQP4-Abs can differentiate and survive in the peripheral circulation. It has been previously shown that autoantibodies producing plasma cells accumulate in peripheral lymphoid organs (34). It would be interesting to investigate which organs blood PB move to during the course of NMO. The levels of IL-6 in the serum and CSF are elevated in NMO compared with HS or CMS patients (27, 28). In this regard, it is of note that blocking IL-6R signaling was found to dramatically reduce the survival of PB ex vivo, which was dependent on the presence of autologous serum containing IL-6. These results suggest that the increase of PB in AQP4-Ab seropositive patients could be attributed to the increased IL-6 in the serum. We also demonstrated that improved PB survival in the presence of exogenous IL-6 was accompanied by up-regulated expression of XBP-1. It is noteworthy that wild-type and $XBP\text{-}1^{-/-}$ B cells start to produce more IL-6 after forced overexpression of XBP-1(s), which results in the operation of a positive feedback loop controlling IgG secretion (31). Treatment with anti–IL-6R is promising because IL-6R blockade could terminate this vicious loop that controls the production of autoantibodies.

It has been reported that NMO patients have higher levels of BAFF in the serum or CSF compared with CMS patients (35). BAFF is also known to support plasma cell differentiation and survival of PB induced in vitro (25). However, in our ex vivo

study, BAFF did not promote the survival of PB, indicating that PB were not a target for BAFF. We speculate that BAFF might specifically act on an early process of plasma cell differentiation and does not have an influence on cells like PB that have entered a later stage.

IL-6R blockade by humanized mAb against IL-6R (tocilizumab) has proven to be useful for treating immune-mediated diseases, including rheumatoid arthritis (36) and Castleman's disease (37). Here we propose that IL-6R–blocking antibody treatment should be considered as a therapeutic option for NMO. Currently, most NMO patients are being treated with corticosteroids in combination with immunosuppressive drugs and plasma exchange (38). Anti-CD20 mAb, which causes B-cell depletion, has also been used for serious cases of NMO. Because the level of B-cell depletion appears to correlate with the suppressive effects of anti-CD20 in NMO (39), it has been argued that B cells are essential for the pathogenesis of NMO, either via acting as antigen-presenting cells or as autoantibody producers. Weber et al. recently reported that activated antigen-specific B cells serve as antigen-presenting cells and polarize proinflammatory T cells in EAE (40), supporting the view that the therapeutic effects of anti-CD20 might be attributable to the depletion of antigen-presenting B cells. Notably, they also cautioned that elimination of $CD20^+$ cells might deplete nonactivated cells as well as regulatory B cells possessing anti-inflammatory potentials. Although the effect of anti-CD20 on AQP4-Ab–secreting cells has not been reported, it is likely that the majority of PB are not affected because they do not express CD20. Consistent with our prediction, anti-CD20 treatment was not effective in aggressive cases of NMO (41, 42). It appears that selective depletion of activated antigen-specific B cells could be a more promising strategy to improve the efficacy of B-cell–targeted therapies for NMO. In this regard, PB-targeting therapy is a promising approach. Given the efficacy of IL-6R blockade in reducing the number of PB ex vivo, we find it very interesting to explore the effect of anti–IL-6R mAb on NMO.

Materials and Methods

Patients and Controls. A cohort of 24 AQP4-Ab seropositive patients was recruited at the Multiple Sclerosis Clinic of the National Center of Neurology and Psychiatry (NCNP). Among these, 16 met the revised NMO diagnostic criteria (3). The other 8 were diagnosed with NMO spectrum disorder (1) because they did not develop both myelitis and optic neuritis (optic neuritis alone in 6 cases; myelitis alone in 2 cases). Seventeen age- and sex-matched CMS patients and 20 HS were enrolled as controls. Serum AQP4-Ab levels were measured by a previously reported protocol by courtesy of Kazuo Fujihara at Tohoku University (Sendai, Japan) (33). All CMS patients had relapsing-remitting MS and fulfilled McDonald diagnostic criteria (43).

At the time of blood sampling, 21 seropositive patients were receiving corticosteroids (prednisolone 5–25 mg/d). Seven of these patients were also being treated with azathioprine (12.5–100 mg/d) or tacrolimus (3 mg/d). Six CMS patients were receiving low-dose corticosteroids without immunosuppressants. None of the seropositive or CMS patients had received IFN-β, i.v. corticosteroids, plasma exchange, or i.v. immunoglobulins for at least 1 mo before blood sampling. Blood sampling during relapse was performed in six seropositive NMO patients before they received intensive therapy starting with i.v. corticosteroids. Five of these patients were followed up further and blood was collected again after they entered remission. Anti-nuclear and/or anti-SS-A Abs were detected in some of the seropositive patients, but none met the diagnostic criteria for SLE or Sjögren syndrome. Demographic features of the patients are presented in Table 1. The study was approved by the Ethics Committee of the NCNP.

Reagents. The following Abs were used in this study: mAbs against CD38, CD19, CD27, CD20, and PE-streptavidin (Beckman Coulter); mAbs against CD180 and BAFF-R (BD Biosciences); mAbs against IL-6R and TACI as well as Abs against BCMA and CD40 (R&D Systems); rabbit anti-human AQP4 antibody (Santa Cruz Biotechnology); FITC-anti-rabbit IgG (Jackson ImmunoResearch Laboratories); and FITC-anti-human IgG antibody (MP Biomedicals). Recombinant proteins of BAFF (ProSpec), APRIL (Abnova), IL-6 (PeproTech), and IL-21 (Invitrogen) were purchased. Propidium iodide (PI) was obtained from Sigma-Aldrich. RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies) was used for cell culture.

Flow Cytometry, Cytology, and Cell Culture. PBMC were separated using density centrifugation on Ficoll-Paque PLUS (GE Healthcare Biosciences). B cells were analyzed and sorted by FACSAria (BD Biosciences). Each B-cell subset was stained with May–Grünwald–Giemsa. To evaluate AQP4-Ab production in vitro, each B-cell subset (1 or 2×10^4) was cultured for 6 d in the medium alone, in the presence of IL-6 (1 ng/mL) or in the presence of IL-6 (1 ng/mL), IL-21 (50 ng/mL), and anti-CD40 (1 μg/mL). Culture supernatants were harvested and analyzed for AQP4-Ab production as described below. To examine the effect of cytokines on the survival of PB, the cells (4×10^3) were cultured in the medium alone or in the presence of BAFF (100 ng/mL), APRIL (300 ng/mL), or IL-6 (1 ng/mL) in 96-well U-bottom plates for 2 d and stained with PI to assess cell survival. In parallel, the cells were cultured for 1 d and harvested to evaluate mRNA expression by qRT-PCR. To assess the effect of IL-6 signaling blockade, PBMC (5×10^5) were preincubated with anti-IL-6R Abs (1 μg/mL) at 4 °C for 20 min, cultured in AIM-V medium (Invitrogen) containing 20% of heat-inactivated serum obtained from each patient in 96-well flat-bottom plates for 2 d, and analyzed by flow cytometry.

Quantitative RT-PCR Analysis. mRNA from each cell subset was isolated according to the manufacturer's instructions using an RNAeasy Kit (Qiagen). RNA was further treated with DNase using the RNase-Free DNase Set (Qiagen) and reverse-transcribed to cDNA using a cDNA synthesis kit (Takara Bio). PCR was performed using iQ SYBR Green Supermix (Takara Bio) on a LightCycler (Roche). RNA levels were normalized to endogenous β-actin (ACTB) for each sample. Primers used are listed in Table S1.

Measurement of Ig Isotypes and Serum IL-6. Secreted IgG in the culture supernatant was quantitated by sandwich ELISA using affinity-purified goat anti-human IgG-Fc (Bethyl Laboratories). Bound IgG was measured according to the manufacturer's instructions. Serum IL-6 was measured by ELISA (R&D Systems) according to the manufacturer's instructions.

AQP4-Ab Detection Assay. Human AQP4-expressing cells were established to detect AQP4-Abs by flow cytometry. A human AQP4 (hAQP4) M23 splice variant from a clone collection (Invitrogen) was amplified by PCR and subcloned into a pIRES-DsRed-Express vector (Clontech). CHO cells (American Type Culture Collection) were transfected with this hAQP4 M23 vector (CHO^{AQP4}) or vector

Table 1. Demographic features

	HS	Seropositive patients	CMS patients
Number	20	24	17
Age	44.7 ± 2.8	47.9 ± 3.2	41.3 ± 3.0
Male:female	5:15	1:23	5:12
Disease duration		12.0 ± 1.6	9.4 ± 2.4
Age of symptom onset		36.1 ± 3.0	31.9 ± 3.4
Relapses in last 2 y		1.4 ± 0.3	0.7 ± 0.2
EDSS score in disease remission		5.0 ± 0.5	2.1 ± 0.6
Other autoantibodies		ANA 13, SS-A 5	ND

Demographic features for HS, AQP4-Ab seropositive patients, and CMS patients. Values are expressed as number or mean ± SEM. ANA, anti-nuclear antibody; ND, not detected; SS-A, anti-SS-A antibody; EDSS, expanded disability status scale.

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control (CHO^{VC}) using FuGENE 6 Transfection Reagent (Roche). After 2 wk of geneticin (Invitrogen) selection, stable clones were established by single-cell sorting. The expression of hAQP4 in the established clones was confirmed using anti-human AQP4 antibody and FITC-anti-rabbit IgG antibody. Reactivity of AQP4-Abs to CHO^{AQP4} was confirmed using seropositive NMO patients' sera diluted at 1:1,000 and FITC-anti-human IgG antibody. To measure the AQP4-Ab activity in culture supernatants, these supernatants were concentrated up to 10 times using an Amicon Ultra 0.5 mL 100K device (Millipore), and 10 μL of the solution was added to 3×10^4 CHO^{AQP4} and CHO^{VC} cells. After incubation on ice for 20 min, cells were washed with sterile PBS containing 1% BSA and stained with FITC-anti-human IgG antibody. After a 10-min incubation on ice, the cells were washed and fixed for 15 min in 2% paraformaldehyde. Then the cells were washed and analyzed by flow cytometry.

Data Analysis. Histogram overlay analysis was performed using Cell Quest software (BD Biosciences). Statistics were calculated using Prism (GraphPad Software). Wilcoxon signed-rank test, Mann–Whitney *U* test, ANOVA, or Spearman's correlation test were also used when appropriate. Post hoc tests were used as a multiple comparison test after confirmation of equal variances by ANOVA.

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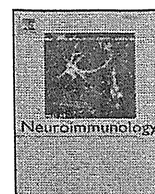

Increase of Ki-67+ natural killer cells in multiple sclerosis patients treated with interferon-β and interferon-β combined with low-dose oral steroids

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ABSTRACT

Interferon-β (IFN-β) is known to expand regulatory CD56^{bright}natural killer (NK) cells in multiple sclerosis (MS). In this cross-sectional study we show that MS patients treated with IFN-β alone or in combination with low-dose prednisolone displayed increased proportion of all NK cell subsets in the active phase of the cell cycle (Ki-67^{+}). There was no difference in NK cell apoptosis markers. In vitro experiments showed that both IFN-β and IFN-β in combination with corticosteroids increased the proportion of Ki-67^{+} NK cells. This study, although limited, shows that treatment with IFN-β affects NK cell cycle without altering NK cell apoptosis in MS patients.

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1. Introduction

Interferon-β (IFN-β) has been used as mainstream treatment in multiple sclerosis (MS) for decades. Early treatment with IFN-β reduces relapse rate and disability (Goodin et al., 2002). High dose short-term intravenous steroids are commonly used to accelerate recovery from acute relapses; however, there is not enough evidence to support a protective effect on long-term disability (Ciccone et al., 2008; Myhr and Mellgren, 2009). Of note, high dose pulses of IVMP had prolonged benefit on gadolinium enhancing lesions in IFN-β treated patients as compared to patients not on treatment with IFN-β (Gasperini et al., 1998). In recent studies pulsed oral methylprednisolone (MP) as add-on therapy to subcutaneous IFN-β1a reduced relapse rate (Sorensen et al., 2009) but was not associated with reduction in long-term disability (Ravnborg et al., 2010). Combination treatment of intramuscular IFN-β1a and intravenous MP (IVMP) did not show significant benefit in relapsing-remitting MS (Cohen et al., 2009). Low-dose oral prednisolone (PDN) as add-on treatment to IFN-β is less commonly used in MS but is considered in clinical practice for patients with reduced response to IFN-β in some countries, including Japan. In a recent study IFN-β1a with either azathioprine or azathioprine with PDN in IFN-β naïve patients was not superior to monotherapy (Havrdova et al., 2009).

Natural killer (NK) cell numbers and function are thought to be altered in MS (Benczur et al., 1980; Kastrukoff et al., 2003; De Jager et al., 2008). NK cells comprise two distinct populations, CD56^{dim} with predominant cytotoxic activity and CD56^{bright} with predominant cytokine producing and possibly immunoregulatory activity (Caligiuri, 2008). To date, there are still contradicting views on the role of NK cells in the pathogenic process underlying MS (Lunemann and Munz, 2008). Reports of reduction of NK cell numbers and function in MS patients and evidence from experimental autoimmune encephalomyelitis (EAE) suggest a protective role of this population (Zhang et al., 1997; Kastrukoff et al., 2003; De Jager et al., 2008). However, this subset can also exert a detrimental role in the inflammatory process within the CNS (Winkler-Pickett et al., 2008). Interestingly, recent work in EAE suggests an organ-specific suppressive function of NK cells towards Th17 cells which are considered by someone to be the main mediators of the inflammatory process in both EAE and MS (Hao et al., 2010).

IFN-β is a type I interferon that can have opposite functional effects on different types of immune cells (Feng et al., 2002). The effects of IFN-β treatment on the adaptive and innate immune response in MS have been thoroughly investigated (Dhib-Jalbut and Marks, 2010). Treatment with IFN-β leads to a reduction of circulating total NK cells (Perini et al., 2000; Hartrich et al., 2003) and the expansion of CD56^{bright} NK cells, suggesting a protective role of this subpopulation (Saraste et al., 2007; Vandenbark et al., 2009). The mechanisms underlying the effects of IFN-β on both total and CD56^{bright} NK cells

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are still not understood. IFN-β treatment does not affect IFN-γ production and in vitro cytotoxicity of NK cells (Kastrukoff et al., 1999; Furlan et al., 2000). An expansion of CD56^{bright} cells is observed after treatment with daclizumab, a monoclonal antibody against the IL-2 receptor α chain (Bielekova et al., 2006). Daclizumab leads to an IL-2 driven activation of CD56^{bright} NK cells with increase in their cytotoxic activity (Martin et al., 2010). Treatment with this monoclonal antibody, either in combination with IFN-β or in monotherapy, is associated with an increased proportion of Ki-67$^+$ NK cells, which are considered proliferating cells (Martin et al., 2010).

In this cross-sectional study we report that in vivo treatment with IFN-β alone or in combination with low-dose PDN leads to a significant increase of NK cells in the active phase of the cell cycle (Ki-67$^+$). We further examined the effects of IFN-β on the ex vivo apoptotic rate of NK cells and did not find any significant difference, although IFN-β plus PDN increased the expression of the anti-apoptotic marker Bcl-2. Finally we demonstrate that in vitro treatment with IFN-β alone or in combination with corticosteroids is associated with increased proportion of Ki-67$^+$ NK cells.

2. Materials and methods

2.1. Subjects

Fifty-three MS patients and 15 healthy volunteers were recruited at the National Center of Neurology and Psychiatry (NCNP) in Kodaira, Tokyo, Japan. Clinical characteristic of patients and healthy subjects are summarised in Table 1a and b. Patients were classified as clinically isolated syndrome (CIS), relapsing-remitting (RR-MS), secondary progressive (SP-MS) or primary progressive (PP-MS) according to international criteria (Polman et al., 2005). Only one patient was diagnosed as pure optic-spinal form of multiple sclerosis (OS-MS), which is more common in the Japanese population (Misu et al., 2002). Patients with neuromyelitis optica were excluded. Twenty-four patients were untreated at the time of sampling (19 RR-MS, 1 CIS, 3 SP-MS, 1 PP-MS) whilst 29 patients were under treatment either with IFN-β (12 RR-MS), IFN-β + PDN (6 RR-MS) or only PDN (9 RR-MS, 1 SP-MS and 1 OS-MS). Five patients were treated with IFN-β1b (Betaferon®) and 7 patients were treated with IFN-β1a (Avonex®) in the IFN-β treated group. Three patients were treated with IFN-β1b (Betaferon®) and 3 patients were treated with IFN-β1a (Avonex®) in the IFN-β + PDN treated group. IFN-β + PDN patients were treated with low-dose steroids because of the persistence of relapses on treatment with IFN-β alone. The duration of the treatment with IFN-β alone was 2.0 (1.5) years and the duration of the treatment with IFN-β + PDN was 0.7 (0.3) years. The average (SD) daily dose of PDN was 4.6 (2.3) mg in the IFN-β + PDN group and 9.0 (4.8) mg in the PDN group. The blood was drawn at variable intervals after IFN-β injections with a range from 12 h to 6 days. All the patients were in the state of remission at the time of the study as determined by history and clinical assessment. Untreated patients had not been given any immunosuppressive medication or corticosteroids for at least 1 month before inclusion in the study.

Written informed consent was obtained from all patients and healthy volunteers and the study was approved by the Ethics Committee of the NCNP.

2.2. Isolation and stimulation of PBMC

Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation with Ficoll-Hypaque Plus (Amersham Biosciences, Uppsala, Sweden). To test the proportion of Ki-67$^+$ cells after culture in vitro, PBMC (0.5×10^6 cells per well in 200 µl final volume) were cultured for 3 days in the presence or absence of IFN-β at increasing concentrations (12.5, 25, 50, 100, 250 ng/ml; 250 ng/ml equivalent to 8,000 IU/ml) and/or dexamethasone (0.01 and 0.1 nM).

2.3. Reagents and flow cytometry

Anti CD3-PC5 and FITC, anti CD56-PE and PC5 monoclonal antibodies (mAbs) were purchased from Immunotech (Marseille, France). Anti CD3-APC, Ki-67-FITC, Bcl-2-FITC, Annexin-V-FITC mAbs and 7-AAD were purchased from BD PharMingen (San Jose, CA, USA). Interferon-β was purchased from Peprotech (Rocky Hill, NJ, USA) and dexamethasone was purchased from Sigma-Aldrich (St. Louis, MO, USA). After staining with the appropriate antibody cells were analysed on an Epics flow cytometer (Beckman Coulter, CA, USA). Stainings with anti CD3-APC, CD56-PE, Annexin-V-FITC and 7-ADD were analysed on FACS

Table 1

a. Clinical characteristics of all the subjects recruited in the study

Subjects	n	Disease type	Mean age (SD)	Sex	Mean disease duration in years (SD)	Annual relapse rate* [mean (SD)]	EDSS [median (SD)]
Healthy controls	15	–	33.5 (7.3)	6 M 9 F	–	–	–
Untreated MS	24	19 RR-MS, 1 CIS, 3 SP-MS, 1 PP-MS	43.1 (11.0)	8 M 16 F	8.3 (7.6)	1.1 (1.8)	1.3 (1.7)
IFN-β treated MS	12	12 RR-MS	29.3 (6.2)	5 M 7 F	4.6 (2.7)	1.9 (1.1)	2.3 (1.7)
IFN-β + PDN treated MS	6	6 RR-MS	40.2 (17.2)	1 M 5 F	9.3 (8.1)	1.3 (2.0)	3.3 (0.3)
PDN treated MS	11	9 RR 1 SP 1 OS	42.3 (9.6)	3 M 8 F	4.1 (3.0)	1.3 (0.8)	3.0 (1.8)

b. Clinical characteristics of treated patients recruited in the study

Subjects	n	Type T	Duration T (years)	Relapse rate	Relapse rate
				BT	AT
IFN-β treated MS	12	IFN-β1a (n=6), IFN-β 1b (n=6)	2.0 (1.5)	1.9 (1.1)	0.1 (0.2)
IFN-β + PDN treated MS	6	IFN-β1a (n=3), IFN-β 1b (n=3)	0.7 (0.3)	1.3 (2.0)	0.1 (0.2)

*Relapse rate before starting treatment in case of IFN-β treated patients. T = treatment; BT = before treatment; AT = after treatment. IFN-β1a = Avonex®; IFN-β1b = Betaferon®.

Calibur (Becton Dickinson, NJ, USA). Weasel flow cytometry software (http://en.bio-soft.net/other/WEASEL.html) was used for data analysis.

2.4. Statistical analysis

We used Kruskall–Wallis test and Dunn's post test for 5-group comparison (healthy controls, untreated MS, IFN-β treated MS, IFN-β + PDN treated MS and PDN treated MS patients) and paired T test for paired samples. Correlations were performed using Spearman test. A p value greater than 0.05 was deemed statistically significant.

3. Results

3.1. IFN-β and (IFN-β + PDN) treated MS patients show increase of circulating Ki-67+ NK cells

We found a significant increase in the proportion of Ki-67$^+$ NK cells in IFN-β and IFN-β + PDN treated patients as compared to the other groups [mean (SD): healthy controls 5.1 (2.1), untreated MS 6.2 (5.0), IFN-β treated MS 15.0 (7.9), IFN-β + PDN treated MS 29.0 (16.2), PDN treated MS patients 5.1 (2.4); $p<0.0001$, Fig. 1]. Ki-67$^+$ total NK cells were significantly increased in IFN-β treated MS patients as compared to HC ($p<0.05$), untreated MS ($p<0.01$) and PDN treated MS patients ($p<0.05$) (Fig. 1B). Similarly, Ki-67$^+$ total NK cells were significantly increased in IFN-β + PDN treated MS patients as compared to HC ($p<0.01$), untreated MS ($p<0.001$) and PDN treated MS patients ($p<0.01$). When we analysed separately CD56^{dim} and CD56^{bright} NK cells, we found that the significant increase in the proportion of Ki-67$^+$ cells was particularly evident in the CD56^{dim} subset [mean (SD): healthy controls 4.5 (2.1), untreated MS 6.1 (6.3), IFN-β treated MS 13.4 (6.8), IFN-β + PDN treated MS 29.6 (17.2), PDN treated MS patients 4.7 (2.6); $p<0.0001$]. Ki-67$^+$ CD56^{dim} NK cells were significantly increased in IFN-β treated MS patients as compared to HC ($p<0.05$), untreated MS ($p<0.01$) and PDN treated MS patients ($p<0.05$) (Fig. 1C). Similar to findings in total NK cells, Ki-67$^+$ CD56^{dim} NK cells were significantly increased in IFN-β + PDN treated MS patients as compared to HC ($p<0.01$), untreated MS ($p<0.01$) and PDN treated MS patients ($p<0.01$). Also the proportion of CD56 ^{bright} Ki-67$^+$ NK cells showed a significantly different distribution between groups ($p=0.005$). Post test analysis showed significant differences in only IFN-β + PDN treated MS patients as compared to HC ($p<0.01$) and untreated MS patients ($p<0.05$) (Fig. 1D).

Our cohort included patients with various treatment durations and we did not have longitudinal data before and after starting treatment. There were no significant differences in disease duration between groups; however, IFN-β treated patients were significantly younger than untreated MS ($p<0.01$) and PDN treated MS patients ($p<0.05$). There was a significant difference in relapse rate between groups ($p=0.03$) and in particular relapse rate was higher in IFN-β treated as compared to untreated MS patients [mean (SD) was 1.9 (1.1) versus 1.1 (1.8), respectively; ($p<0.05$)] (Table 1b). The relapse rate decreased in both IFN-β treated and IFN-β + PDN treated MS patients after treatment [mean (SD) 1.9 (1.1) before treatment and 0.1 (0.2) after treatment in IFN-β treated MS patients; 1.3 (2.0) before treatment and

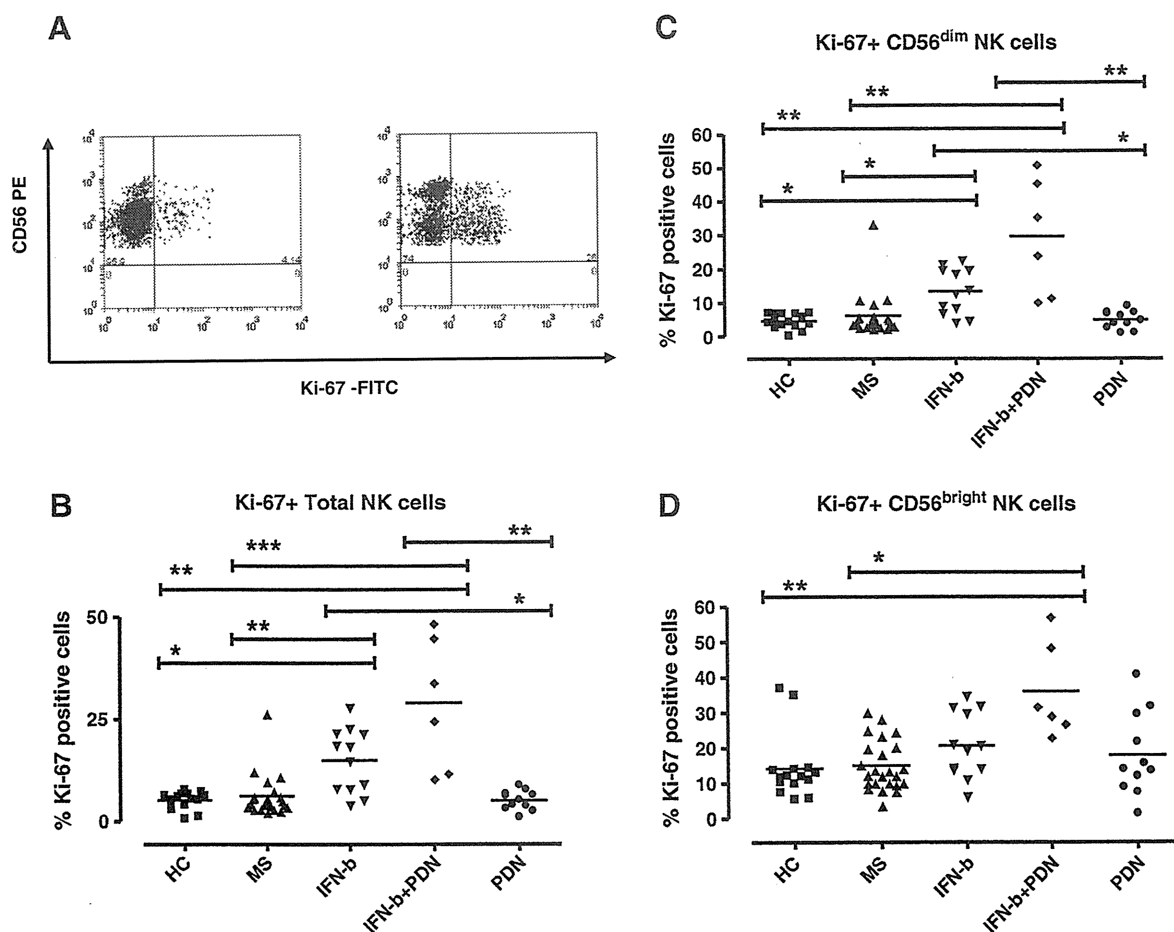

Fig. 1. Frequency of Ki-67$^+$ cells is increased in NK cells from both IFN-β treated and IFN-β + PDN treated patients. (A) Representative expression pattern of Ki-67 versus CD56 in one healthy control (left) and one IFN-β treated MS patient (right) in gated CD3$^-$ lymphocytes. (B–D) Frequency of Ki-67$^+$ total NK cells (B), CD56^{dim} NK cells (C), CD56^{bright} NK cells (D) in the five groups: healthy controls (HC), untreated MS patients (MS), IFN-β treated MS patients (IFN-b), IFN-β and prednisolone treated MS patients (IFN-b + PDN) and prednisolone only treated (PDN) treated MS patients. ***$p<0.001$, **$p<0.01$ and *$p<0.05$; Kruskall–Wallis and Dunn's post test.

0.1 (0.2) after treatment in IFN-β + PDN treated MS patients] (Table 1b). There were no correlations between the relapse rate and the proportion of circulating NK cells. There were no correlations between the percentage of Ki-67^{+} total, CD56^{dim} or CD56^{bright} NK cells and age, relapse rate, EDSS, duration or type of treatment with IFN-β.

3.2. The number of Ki-67^{+} NK cells correlates with reduced proportion of total and CD56^{dim} NK cells in untreated and IFN-β treated MS patients but not in normal controls

As reported previously by our group (Takahashi et al., 2001), there was no difference in the proportion of circulating CD3^{-}CD56^{+} total NK and CD56^{dim} NK cells between untreated and treated MS patients and healthy controls (data not shown). CD56^{bright} NK cells tended to be increased in IFN-β (0.77 ± 0.71%) and IFN-β + PDN treated MS patients (1.15 ± 0.69%) as compared to HC (0.51 ± 0.25%) and untreated MS patients (0.45 ± 0.20%), but there was no significant difference comparing the four groups.

Untreated MS and IFN-β treated MS patients showed a striking inverse correlation between the proportions of total NK cells and the proportions of Ki-67^{+} total NK cells ($p = 0.0052$ and $p = 0.028$, respectively; Fig. 2). A negative correlation was evident also between the proportions of circulating CD56^{dim} NK cells and the proportions of Ki-67^{+} CD56^{dim} NK cells in both untreated and IFN-β treated MS patients ($p = 0.0037$ and $p = 0.005$, respectively; data not shown). There was no significant correlation in healthy controls, IFN-β + PDN and PDN treated MS patients (data not shown).

When we analysed CD56^{bright} NK cells, the negative correlation between the proportion of circulating CD56 ^{bright} NK cells and the proportion of Ki-67^{+} CD56 ^{bright} NK cells was present in only untreated MS patients ($p = 0.034$; data not shown).

3.3. IFN-β treatment does not affect the apoptotic rate of NK cells in MS patients

To determine whether the increased Ki-67 expression of NK cells in IFN-β and IFN-β + PDN treated patients was associated with alteration of apoptotic markers, we first determined the intracellular expression of the anti-apoptotic protein Bcl-2. IFN-β treated patients showed a tendency to increased Bcl-2 expression in NK cells, although this was not statistically significant. IFN-β + PDN treated patients showed a significant increased frequency of expression of Bcl-2 in total NK, CD56^{dim} and ^{bright} NK cells as compared to MS patients ($p < 0.05$; Fig. 3A, B and C, respectively). This statistical difference was not confirmed analysing the MFI of Bcl-2 expression (data not shown).

We then examined the ex vivo proportion of apoptotic NK cell subsets by staining freshly isolated PBMC with Annexin-V and 7-AAD. Treatment with IFN-β did not alter the proportion of apoptotic (Annexin-V positive and 7-AAD negative) total NK cells as compared to untreated MS and HC (Fig. 3D). Similarly, there was no difference in the proportion of apoptotic CD56^{dim} and ^{bright} NK cells (data not shown). Unfortunately it was not possible to test IFN-β + PDN treated MS patients for Annexin-V and 7-AAD staining due to time limitations.

3.4. In vitro IFN-β increases the number of Ki-67^{+} NK cells

To determine whether IFN-β and/or corticosteroids can directly stimulate an increase of Ki-67^{+} NK cells, we performed in vitro experiments on PBMC freshly isolated from normal subjects. PBMC were cultured either without or with increasing high IFN-β concentrations (range: 12.5–250 ng/ml; 12.5 ng/ml equivalent to 400 IU/ml). These concentrations are above levels detected on average in MS

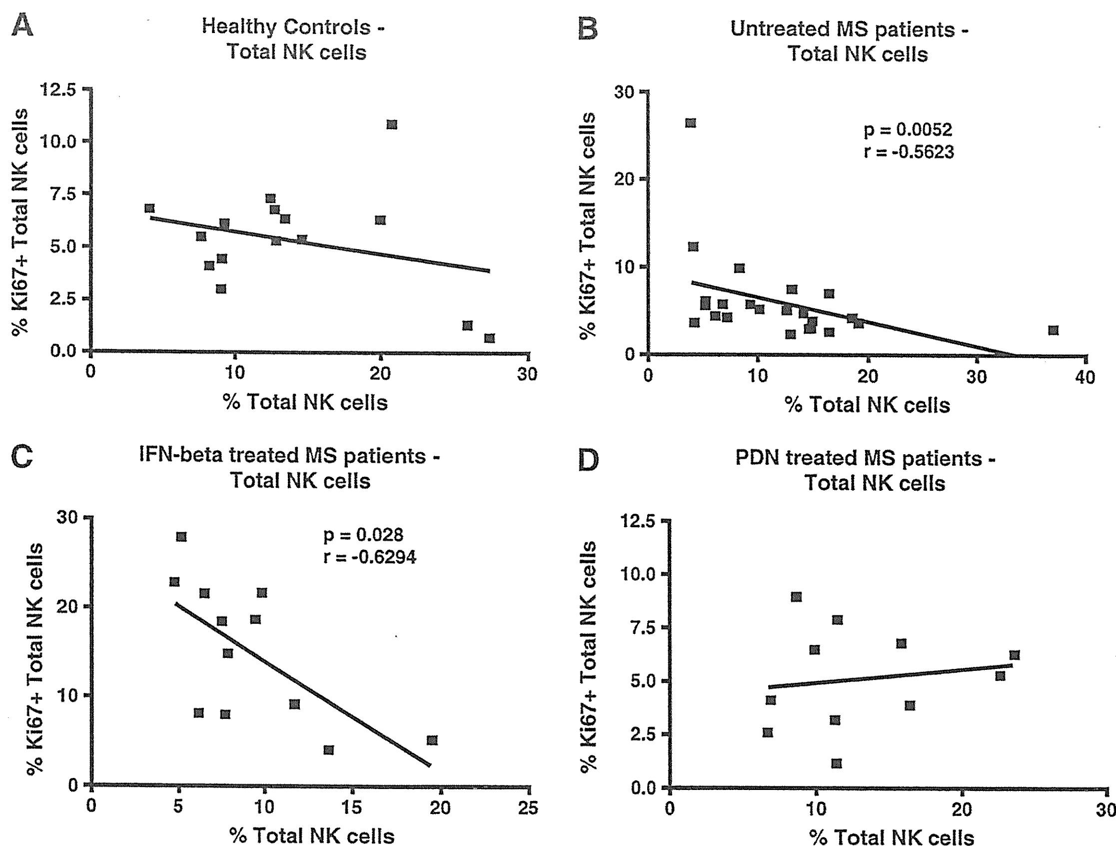

Fig. 2. The proportion of Ki-67^{+} NK cells is inversely correlated with the proportion of circulating cells in untreated MS and IFN-β treated MS but not in HC. (A) The proportion of total NK cells and Ki-67^{+} NK cells does not show any statistically significant correlation in either healthy controls (A) or PDN treated MS patients (D). The proportion of total NK cells and Ki-67^{+} NK cells is inversely correlated in MS patients (B) and IFN-β treated MS patients (C). Spearman test.

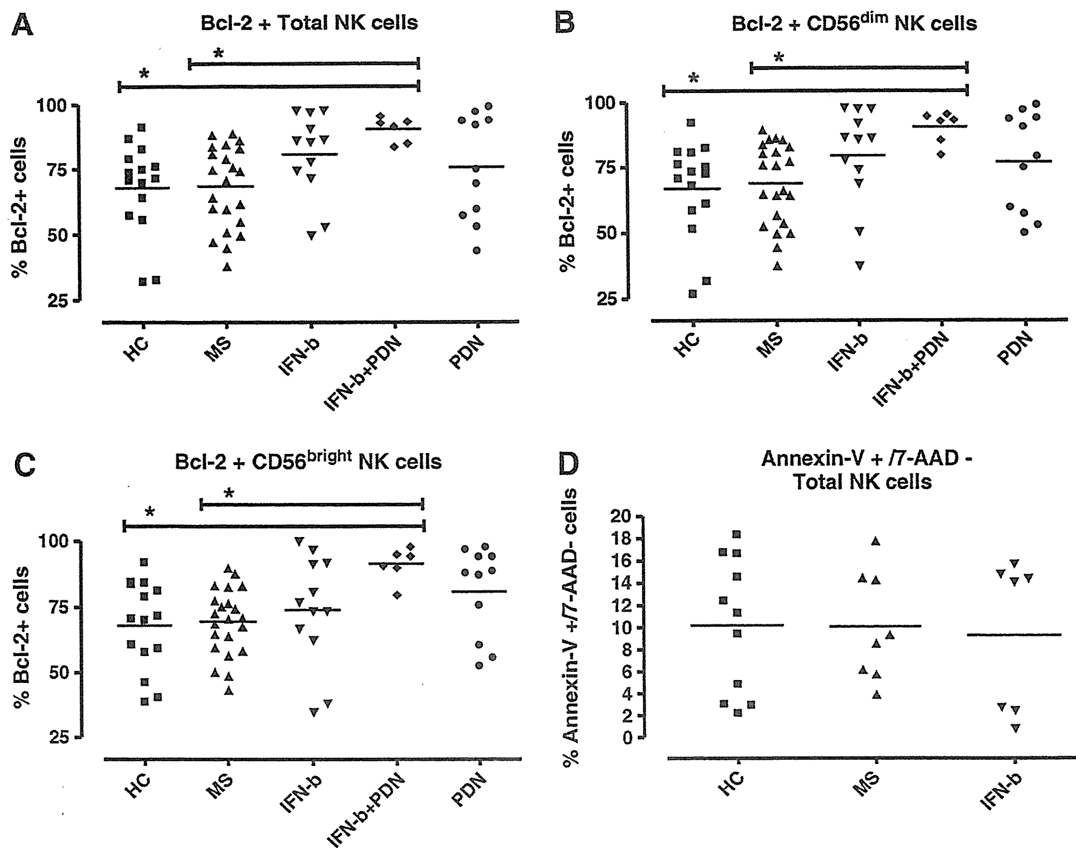

Fig. 3. Effects of IFN-β and IFN-β + PDN treatment on apoptotic markers. (A–C) The intracellular expression of Bcl-2 is significantly increased in IFN-β + PDN treated patients as compared to HC and untreated MS. (D) There was no difference in the proportion of Annexin-V^+/7-AAD^- NK cells in HC, MS and IFN-β treated MS patients. *$p<0.05$; Kruskall–Wallis and Dunn's post test.

patients under treatment with IFN-β. Serum IFN-β levels in MS patients treated with intramuscular IFN-β1a vary between 64 and 86 IU/ml after 3 or 6 months of treatment (Khan and Dhib-Jalbut, 1998). We found that in vitro IFN-β at the highest concentration induced marked increase in NK cell apoptosis (data not shown). IFN-β significantly increased the proportion of Ki-67^+ NK cells at all tested concentrations but 12.5 ng/ml (Fig. 4A; $n = 10$). Similar results were found with purified NK cells (data not shown). We then examined the in vitro effect of IFN-β + dexamethasone (DEX) (Fig. 4B; $n = 4$). Cells were cultured either without or with 25 ng/ml of IFN-β and/or DEX (0.01 or 0.1 nM, equivalent to 3.925 and 39.25 pg/ml, respectively). We found a significant increase of Ki-67^+ NK cells in IFN-β + DEX culture at the concentration of 0.1nM of DEX ($p = 0.046$) and a trend at the lower concentration of 0.01 nM ($p = 0.07$) as compared to medium. There was no difference after culture with only DEX at either 0.1 or 0.01 nM concentration.

4. Discussion

Here we show that treatment with IFN-β and IFN-β + PDN in MS patients leads to expansion of NK cells in the active phase of the cell cycle (Ki-67^+). This expansion was most evident in $CD56^{dim}$ NK cells. We also show that treatment with IFN-β does not significantly affect the apoptotic rate of NK cells. Of note, our patients were treated with either IFN-β or IFN-β combined with low-dose oral steroids and did not receive regular high dose pulses of steroids. In vitro experiments showed that IFN-β either alone or in combination with corticosteroids can increase the proportion of Ki-67^+ NK cells, suggesting a direct effect of IFN-β on NK cells.

IFN-β leads to reduction of circulating total NK cells and expansion of $CD56^{bright}$ NK cells in MS patients (Perini et al., 2000; Hartrich et al., 2003; Saraste et al., 2007; Vandenbark et al., 2009). Moreover, it increases activated ($CD69^+$) NK cells but does not affect NK function (Kastrukoff et al., 1999; Furlan et al., 2000; Hartrich et al., 2003). Of note, the in vivo effects observed in MS patients do not necessarily correspond to the in vitro effects of IFN-β on NK cells. IFN-β in vitro inhibits IL-2 induced proliferation of NK cells and increases their IFN-β production (Hunter et al., 1997). To date, the mechanisms underlying the effects of IFN-β treatment on NK cell populations in MS are not completely understood. Two potential mechanisms can be hypothesised to explain the reduction of circulating NK cells associated with IFN-β treatment: (1) increase in turnover and apoptosis of either activated or resting NK cells; (2) redistribution of NK cells with migration to the peripheral tissues and particularly the CNS. These mechanisms could target either total NK cells or selectively $CD56^{dim}$ and $CD56^{bright}$ subsets. A gradual change in total NK numbers during treatment with IFN-β has been observed in MS patients, suggesting a gradual shift in immune homeostasis (Perini et al., 2000; Hartrich et al., 2003). Maximal reduction in NK cell numbers occurs 8 h after administration with persistent reduction at 3 months and return to baseline levels at 6 months (Hartrich et al., 2003).

Our study showed that IFN-β is associated with increased frequency of Ki-67^+ NK cells in a cross-sectional cohort of MS patients. Ki-67 is a nuclear antigen that is expressed exclusively in the active stages of the cell cycle, namely, late G1, S, G2 and M phases (Gerdes et al., 1984). The expression of Ki-67 starts at the first S phase, is present in mitosis and decreases in G1 (Lopez et al., 1991). The increased expression of Ki-67 in NK cells may reflect increased in vivo proliferation or accumulation of NK cells in one specific phase of the cell cycle but G0.

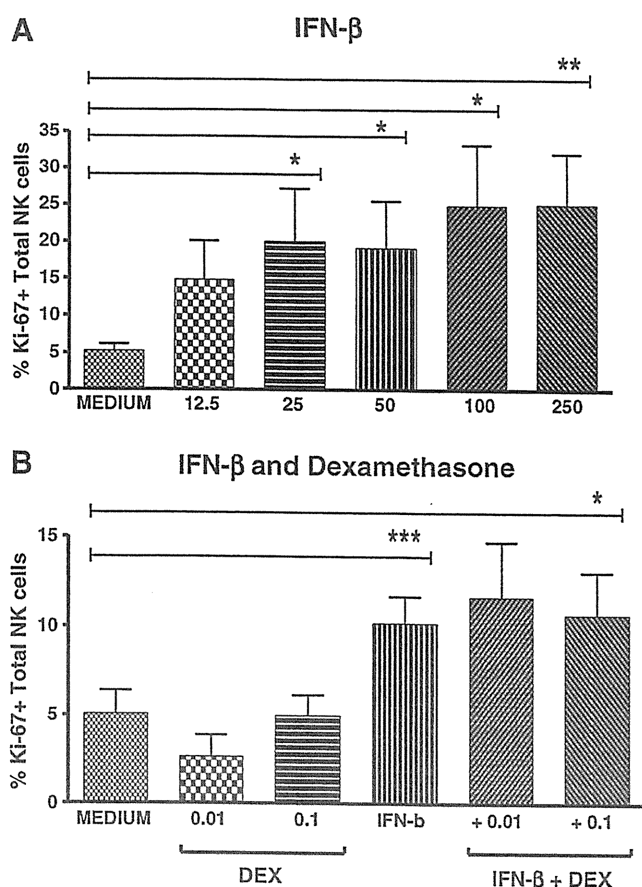

Fig. 4. In vitro effects of IFN-β and IFN-β + dexamethasone (DEX) on Ki-67$^+$ NK cells. PBMC were isolated from normal subjects and cultured for 3 days in medium without or with IFN-β and/or DEX at different concentrations. (A) IFN-β significantly increased the proportion of Ki-67$^+$ NK cells ($n=10$) as compared to medium (12.5 ng/ml, $p=0.06$; 25 ng/ml, $p=0.05$; 50 ng/ml, $p=0.04$; 100 ng/ml, $p=0.03$; 250 ng/ml, $p=0.01$). (B) Cells were cultured either without or with 25 ng/ml of IFN-β and/or DEX (0.01 or 0.1 nM respectively). IFN-β + DEX significantly increased the proportion of Ki-67$^+$ NK cells at the concentration of 0.1 nM and showed a trend at the concentration of 0.01 nM ($n=4$; $p<0.05$ and $p=0.07$, respectively). In this set of experiments IFN-β but not DEX alone significantly increased the proportion of Ki-67$^+$ NK cells as compared to medium ($p \leq 0.001$). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; paired T test.

Our study showed that CD56^{dim} NK cells as well as the CD56^{bright} subset are affected by IFN-β treatment. Indeed, the increase in Ki-67$^+$ NK cells was mostly evident in the CD56^{dim} subsets. CD56^{bright} are considered precursors of CD56^{dim} NK cells (Caligiuri, 2008). The two subsets exert distinct functions and may have different roles in regulating the immune response in the periphery and/or in the CNS. The increase of Ki-67$^+$ total NK cells may therefore reflect the expansion of CD56^{bright} subset that has matured into CD56^{dim}. It is still not clear whether the expansion of CD56^{bright} cells in IFN-β treated patients reflects the correction of an underlying defect of NK cell homeostasis in MS patients or whether it is only a physiological effect of IFN-β on NK cells. Moreover, it is not known whether the expansion of CD56^{bright} NK cells is associated with clinical response to IFN-β treatment and can be considered a marker of its therapeutic effect. We did not find any correlation between the proportion of Ki-67$^+$ NK cells and clinical parameters in our study.

This is the first study to examine the effects of combined treatment with IFN-β and corticosteroids on NK cells. Our in vivo observations suggest an increased proportion of Ki-67$^+$ NK cells in IFN-β + PDN treated patients as compared to IFN-β alone but there were no statistically significant differences between the two groups. Similarly, our in vitro observations did not support a prominent effect of corticosteroids over IFN-β in increasing the proportion of Ki-67$^+$ NK cells.

NK cell homeostasis is maintained through the balance between proliferation and cellular loss. We did not find an increased rate of NK apoptosis in IFN-β treated MS patients. IFN-β induced apoptosis of unfractionated peripheral blood lymphocytes (PBLs) in MS patients (Gniadek et al., 2003). However IFN-β treated MS patients showed reduction and normalisation of ex vivo T cell apoptosis (Garcia-Merino et al., 2009). It is likely that in vivo IFN-β treatment exerts different effects on the apoptosis of different cell populations. We observed an increased expression of the anti-apoptotic molecule Bcl-2 in IFN-β + PDN treated patients, suggesting a possible synergistic effect of IFN-β and steroids in preventing NK cell apoptosis. Corticosteroids usually induce apoptosis of immune cells (Wyllie, 1980). However, high dose methylprednisolone (MP) induced unfractionated PBL and T cell apoptosis but did not have any effect on the rate of apoptotic NK cells in MS patients (Leussink et al., 2001). Of note, the observed increased Bcl-2 expression does not necessarily correlate with reduced ex vivo apoptosis of NK cells and could represent the effect of changes in the cytokine milieu secondary to the combined treatment of IFN-β + PDN (Graninger et al., 2000).

We found that the proportion of Ki-67$^+$ NK cells was inversely correlated with the proportion of circulating NK cells in both untreated and IFN-β treated MS patients. This is consistent with an inverse correlation between NK cell numbers and expression of NK activation markers (CD69$^+$ NK cells) (Hartrich et al., 2003). NK cell activation requires migration into the tissues and results in reduced NK frequency in the periphery. We hypothesise that IFN-β treatment may be associated with increased migration of activated and proliferating NK cells to the periphery and/or the CNS. Since the inverse correlation between NK cell proportion and Ki-67$^+$ NK cells was also observed in untreated MS patients, this phenomenon may represent an attempt of the immune system to regulate the inflammatory process. In this instance, IFN-β treatment would enhance a protective mechanism that occurs also in untreated MS patients in remission.

In conclusion, here we demonstrate that treatment with IFN-β in either monotherapy or combination with corticosteroids increases the proportion of NK cells in the active phase of the cell cycle both in vivo and in vitro. We hypothesise that increased proliferation and migration of NK cells to the peripheral tissues may be involved in the disease-modifying effects of IFN-β in MS patients.

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Mucosal-associated invariant T cells regulate Th1 response in multiple sclerosis

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Abstract

Mucosal-associated invariant T (MAIT) cells are innate T cells expressing an invariant Vα7.2-Jα33 T-cell antigen receptor α chain and are enriched in mucosal-associated lymphoid tissues. Although the regulatory role of MAIT cells in experimental autoimmune encephalomyelitis has been determined, their role in multiple sclerosis (MS) has not been elucidated. In the present study, the character of MAIT cells in the peripheral blood of MS patients was analyzed. Compared with healthy controls, the frequency of MAIT cells in peripheral blood was significantly reduced in MS patients in remission and even more profoundly reduced in those with relapse. The frequency of MAIT cells reflected the disease activity, as they were reduced significantly in patients with active disease compared with stable patients, and when blood samples from patients undergoing attack were analyzed 2–3 months later, the frequency significantly increased in parallel with clinical recovery. The frequency of MAIT cells positively correlated with the frequency of $CD4^+$ invariant NKT cells and of $CD56^{bright}$ NK cells in healthy controls but not in MS patients. This suggests the existence of an immune-regulatory link between MAIT cells and these other cell populations with disruption of this cross talk in MS. Moreover, MAIT cells showed a suppressive activity against IFN-γ production by T cells *in vitro*. This suppression required cell contact but was independent of IL-10, inducible co-stimulator or the presence of B cells. Taken together, these results suggest an immune-regulatory role of MAIT cells in MS through suppression of pathogenic T_h1 cells.

Keywords: CD161, immune regulation, IFN-γ, MR1, NKT cells

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) (1). Whereas the cause of MS is multifactorial, a central role has been suggested for autoimmune responses against the myelin component of the CNS (2). This idea is strongly supported by the results of clinical trials with altered peptide ligands. Administration of an altered peptide ligand of myelin basic protein induced MS exacerbation in some patients and this exacerbation was accompanied by an increase in IFN-γ-producing T cells cross-reactive to the original antigen (3). Furthermore, the importance of immune cell entry into the CNS was shown by treatment using a mAb against very late activation-4 (4). In addition, the immunogenetic background of MS and data from animal models also support its autoimmune nature. Currently, the pathological roles of CNS myelin-reactive helper T cells that produce IFN-γ (T_h1) and/or IL-17 (T_h17) are receiving substantial attention (1, 2). However, growing evidence has shown that the immunopathology of MS is more complicated, and the importance of a balance between pathogenic cells and immune-regulatory cells has also been suggested (1, 5). For instance, it was reported that NK cells exhibited suppressive activity against pathogenic T_h1 cells specific to myelin basic protein, but the suppressive property of NK cells was lost in MS patients with relapse (6, 7). In addition, the function of $CD4^+$ invariant natural killer T (iNKT) cells was reported to be biased toward T_h2, an immunosuppressive phenotype, in MS patients in remission but not in those with relapse (8). Moreover, a functional defect in the suppressive activity of $CD4^+CD25^{high}$ regulatory T cells in MS patients was reported (9). These findings suggest that functional defects in immune-regulatory cells are related to the development and/or relapse of MS.

Mucosal-associated invariant T (MAIT) cells are innate T cells first identified among human CD4/CD8 double-negative (DN) T cells as a novel cell population bearing an invariant T-cell antigen receptor (TCR) α chain distinct from Vα24 iNKT cells (10, 11). MAIT cells express a canonical Vα7.2-Jα33 TCR α chain in preferential combination with Vβ2 and Vβ13 in the human and are restricted by MR1, a major histocompatibility complex class Ib molecule expressed on bone marrow-derived cells (12). The

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unique features of MAIT cells are that they are enriched in intestinal lamina propria and their development and peripheral expansion are dependent on the presence of B cells and commensal flora (12–14). Little is known about the role of MAIT cells in health or in disease states, but a disease-suppressive role of this cell population was reported in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (15). Moreover, using PCR single-strand conformational polymorphisms, MAIT cells were found to infiltrate MS lesions (16). However, a detailed picture of the role of MAIT cells in human autoimmune diseases including MS does not yet exist.

In this study, we show that the frequency of MAIT cells in peripheral blood is reduced in MS patients and that their frequency reflects the disease activity of MS. In addition, we found that the frequency of MAIT cells positively correlates with those of $CD4^+$iNKT cells and $CD56^{bright}$ NK cells in healthy subjects but not in MS patients, suggesting that MAIT cells may regulate the immune system in concert with these cell populations to prevent MS. Moreover, we show a suppressive role of MAIT cells against IFN-γ production from T cells and suggest a disease-suppressive role for MAIT cells in MS. This is the first report to demonstrate a role for MAIT cells in human autoimmune diseases.

Materials and methods

Subjects

Thirty-two patients with relapsing–remitting MS [age: 40.2 ± 1.7 (mean ± SE)] and 16 age- and sex-matched healthy controls (HC) (age: 40.4 ± 2.6) were examined. The diagnosis of MS was established according to the 2005 version of the McDonald's criteria (17, 18), and patients with neuromyelitis optica were not included. Among the 32 MS patients, 25 (age: 40.7 ± 2.0) were in remission and 7 (age: 38.1 ± 3.2) were in an acute phase of relapse. None of the patients in remission was taking any medications affecting the immune system, such as IFN-β, corticosteroids or immunosuppressants. Among the seven patients in relapse, four were free of medication, two were being treated with oral prednisolone and one was being treated with mizoribine. This study was approved by the Ethical Committee of the National Institute of Neuroscience and written consent was obtained from all subjects.

Flow cytometry

Fresh peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) separation and then stained with combinations of the following mAb against human cell surface antigens: FITC-anti-TCR-γδ mAb, FITC-anti-TCR-Vα24 mAb, phycoerythrin (PE)-anti-CD161 mAb, PE-anti-TCR-Vβ11 mAb, PE-Texas Red-anti-CD3 mAb, PE-anti-CD4 mAb, allophycocyanin (APC)-anti-CD56 mAb (all from BeckmanCoulter, Brea, CA, USA), FITC-anti-CD19 mAb, Peridinin chlorophyll protein-anti-CD5 mAb, Peridinin chlorophyll protein-anti-CD8α mAb, APC-anti-CC chemokine receptor (CCR) 5 mAb, -mouse IgG1, APC-Cy7-anti-CD4 mAb and APC-Cy7-anti-CD3 mAb (all from BD Biosciences, Franklin Lakes, NJ, USA). FITC-anti-CCR6 mAb and -mouse IgG1 were purchased from R&D Systems (Minneapolis, MN, USA). Staining of biotin-conjugated 3C10 mAb (13) was visualized using streptavidin-PE-Cy7 (BD Biosciences). Cells were analyzed on an FACS Aria flow cytometer (BD Biosciences) with FloJo software (Tree Star, Ashland, OR, USA).

Intracellular cytokine staining

PBMC (5×10^5 cells per well in 24-well culture plates) were stimulated with 50 ng ml^{-1} phorbol-myristate-acetate (PMA) (Sigma, St Louis, MO, USA) and 500 ng ml^{-1} ionomycin (IM) (Sigma) for 4 h at 37°C in 5% CO_2. Monensin (BD Bioscience) was added in the last 2 h of culture at a concentration of 2 μM. After staining the cell surface antigens, intracellular cytokines were stained using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Bioscience) and FITC-anti-IFN-γ (BeckmanCoulter), FITC-anti-IL-17, APC-anti-IL-4 or APC-anti-IL-10 mAbs or their isotype control antibodies (all from eBiosciences, San Diego, CA, USA). Cells were analyzed on an FACS Aria flow cytometer with FloJo software.

Cell culture

To analyze cytokine production from MAIT cells *in vitro*, MAIT cells ($CD5^+CD19^-TCR\text{-}\gamma\delta^-CD161^{high}3C10^+$) or other T cells ($CD5^+CD19^-TCR\text{-}\gamma\delta^-CD161^-3C10^-$) were isolated from the PBMC of HC using a FACS Aria cell sorter and cultured in 96-well flat-bottom plates at 1×10^5 cells per well with RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.05 mM 2-mercaptoethanol, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were stimulated with immobilized anti-CD3 mAb (5 μg ml^{-1}, clone OKT3; American Type Culture Collection, Manassas, VA, USA) and soluble anti-CD28 mAb (1 μg ml^{-1}, clone CD28.2; BeckmanCoulter) or PMA (50 ng ml^{-1}) and IM (500 ng ml^{-1}) for 48 h. IFN-γ, IL-4 and IL-10 in the supernatant were quantified using an ELISA Kit (BD Bioscience). IL-17 was measured using an ELISA Kit purchased from R&D Systems.

To deplete MAIT cells from the PBMC of HC and MS patients, $CD5^+CD19^-TCR\gamma\delta^-CD161^{high}3C10^+$ cells were depleted using a FACS Aria cell sorter. Control PBMC were also stained with the same combination of mAbs and passed through the cell sorter without depletion of MAIT cells. Cells were cultured in 96-well flat-bottom plates at 2×10^5 cells per well and stimulated with 2 μg ml^{-1} of PHA (Sigma). Cytokine concentrations in the supernatant at 48 h of culture were measured using the ELISA kits described above. In some experiments, MAIT cells (1×10^5 cells per well) isolated using the cell sorter were added back into PBMC before stimulation, from which MAIT cells had been depleted. In this add-back experiments, MAIT cells were depleted from PBMC using anti-PE-CD161 mAb and anti-PE-magnetic beads (Miltenyi Biotec, Gladbach, Germany). To block cell contact between MAIT cells and other cells, transwell inserts (Corning, Corning, NY, USA) were used. For blocking experiments, anti-IL-10 mAb (10 μg ml^{-1}), anti-inducible co-stimulator (ICOS)-ligand (L) mAb (10 μg ml^{-1}) (both from eBioscience), anti-transforming growth factor (TGF)-β mAb (10 μg ml^{-1}; R&D systems) or their isotype control antibodies were added to the culture. Anti-CD19-magnetic beads (Miltenyi Biotec) were used to deplete B cells from PBMC.

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Quantification of cytokine mRNA

Total RNA was extracted from isolated MAIT cells or control T cells using RNeasy spin columns (QIAGEN, Germantown, MD, USA) and reverse transcribed into complementary DNA using Primescript reverse transcriptase (Takara, Ohtsu, Japan). Levels of IFN-γ and IL-17 mRNA were measured by quantitative PCR using an SYBR Premix Ex Taq Kit (Takara) on a LightCycler1.5 (Roche, Basel, Switzerland). Expression levels relative to those of β-actin are presented. The primer pairs used were as follows: IFN-γ forward, 5′-ACAGG-GAAGCGAAAAAGGAGTCAG-3′ and IFN-γ reverse, 5′-CAT GGGATCTTGCTTAGGTTGG-3′; IL-17 forward, 5′-CCAG-GATGCCCAAATTCTGAGGAC-3′ and IL-17 reverse, 5′-CAA GGTGAGGTGGATCGGTTGTAG-3′ and β-actin forward, 5′-CACTCTTCCAGCCTTCCTTCC-3′ and β-actin reverse, 5′-GCGTACAGGTCTTTGCGGATG-3′.

Results

MAIT cells are reduced in the peripheral blood of MS patients and reflect disease activity

Previously, $CD161^{high}$ 3C10 (Vα7.2)-positive cells have been reported to represent MAIT cells in adult human peripheral blood (13, 19, 20). Therefore, we used this definition of MAIT cells to analyze the frequency of MAIT cells in peripheral blood by flow cytometry in HC and MS patients in remission (MS remission) or in relapse (MS relapse). MAIT cells could be identified as a distinct cell population bearing a $V\alpha7.2^{+}CD161^{high}$ phenotype in all subjects (Supplementary Figure 1A is available at *International Immunology* Online). Representative profiles of a HC and an MS relapse are shown in Fig. 1(A). In HC, the frequency of MAIT cells among total αβT cells was 3.79 ± 0.52% (mean ±SEM). In MS remission, the frequency of MAIT cells was 2.33 ± 0.39%, which was significantly lower than that in HC (Fig. 1B). The frequencies of $V\alpha7.2^{+}CD161^{low}$ and $V\alpha7.2^{+}CD161^{-}$ or $V\alpha7.2^{-}CD161^{high}$ populations were not different between HC and MS patients, suggesting that the reduced frequency of MAIT cells in MS patients was not simply due to down-modulation of the Vα7.2 TCR or CD161 molecules in MAIT cells in MS patients. In addition to the DN population, within which MAIT cells were first identified, MAIT cells include also CD4, CD8αα and CD8αβ populations (13). Since all the CD4, CD8αβ, CD8αα and the DN MAIT cell sub-populations were reduced in MS patients compared with HC, the decrease in MAIT cell frequency was not attributed to reduction of a certain sub-population of MAIT cells (Fig. 1C). The frequency of total αβT cells among PBMC was not different between HC, MS remission and MS relapse (61.7 ± 4.7, 65.8 ± 1.9 and 67.85 ± 3.2%, respectively).

The decrease in the frequency of MAIT cells was more profound in MS relapse (0.87 ± 0.24%) (Fig. 1B). MS patients who had at least one attack or had been found to have an active lesion by magnetic resonance imaging within 1 year had significantly lower numbers of MAIT cells compared with patients stable for more than a year (Fig. 1D). Furthermore, when MS relapse patients were followed up for 2–3 months after the attack, the frequency of MAIT cells significantly increased along with the clinical recovery

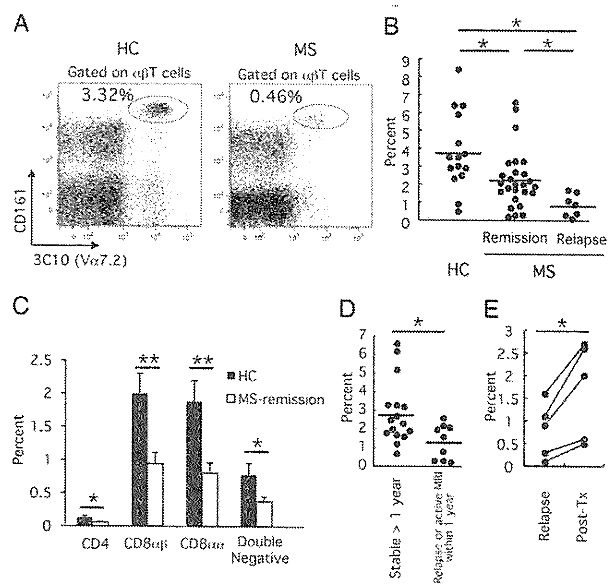

Fig. 1. Frequency of MAIT cells among αβT cells in peripheral blood. (A) Representative flow cytometry profiles of $CD3^{+}TCR\text{-}\gamma\delta^{-}$ cells in a HC (left) and in an MS relapse (right). (B) Frequency of MAIT cells among αβT cells in HC (*N* = 16), MS remission (*N* = 25) and MS relapse (*N* = 7). Each symbol represents the value of one individual. Horizontal bars indicate the means. $*P < 0.05$ (Kruskal–Wallis *H*-test followed by Mann–Whitney *U*-test with Bonferroni correction). (C) Frequency of CD4, CD8αβ, CD8αα and DN MAIT cell sub-population among total αβT cells in HC and MS remission. Error bars represent the SEM. $*P < 0.05$, $**P < 0.01$ (Mann–Whitney *U*-test). Since the proportion of MAIT cells in some patients with MS remission and most of those with MS relapse was too low to assess precisely the frequency of each MAIT cell sub-population, they were not included in the analysis. (D) Frequency of MAIT cells in MS patients stable for >1 year and those who had at least one clinical attack or had been found to have active magnetic resonance imaging lesions within 1 year. $*P < 0.05$ (Mann–Whitney *U*-test). (E) Frequency of MAIT cells in five patients analyzed at an acute phase of relapse and 2–3 months after steroid therapy (Post-Tx). $*P < 0.05$ (Wilcoxon *t*-test).

(Fig. 1E). These results indicate that the frequency of MAIT cells in peripheral blood is reduced in MS remission and reduced even more profoundly in MS relapse, and the frequency reflected disease activity.

The positive correlations in the frequency of MAIT cells with those of $CD4^{+}$iNKT cells and $CD56^{bright}$ NK cells are lost in MS.

Since several other innate lymphocytes such as $CD4^{+}$iNKT cells and $CD56^{bright}$ NK cells are believed to participate in the regulation of MS (8, 21), we next examined the correlations of the frequency of MAIT cells with the frequencies of those innate lymphocytes. As shown in Fig. 2 (upper panels), positive correlations between the frequencies of MAIT cells and those of $CD4^{+}$iNKT cells and $CD56^{bright}$ NK cells were observed in HC. In MS patients, however, the frequency of $CD56^{bright}$ NK cells was decreased along with MAIT cells (Fig. 2, lower right panel). In the case of $CD4^{+}$iNKT cells, the positive correlation with MAIT cells that was observed in HC was disrupted in MS (Fig. 2, lower left panel).

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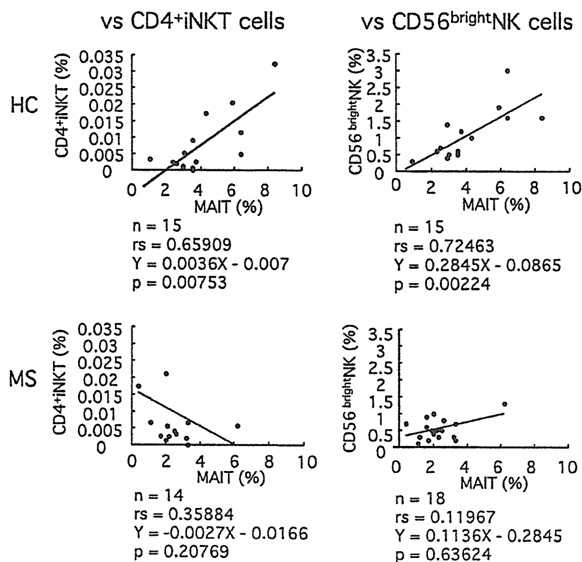

Fig. 2. Correlation of the frequency of MAIT cells with the frequency of $CD4^+$iNKT cells and $CD56^{bright}$ NK cells. The frequency of MAIT cells was plotted against the frequency of $CD4^+$iNKT cells ($CD3^+CD4^+$TCR-$V\alpha24^+$-$V\beta11^+$) among total T cells (left panels) or that of $CD56^{bright}$ NK cells ($CD3^-CD56^{bright}$) among total lymphocytes (right panels) in HC (upper panels) or in MS remission (lower panels). Correlations were analyzed using Spearman's correlation.

Characterization of MAIT cells in HC and MS

To further characterize MAIT cells, we analyzed the expression of chemokine receptors important for CNS invasion. Compared with other T cells, MAIT cells exhibited higher expression of CCR5 and CCR6 (Fig. 3A, top panels), although the expression levels were not different between HC and MS (Fig. 3A, lower panels). We next assessed cytokine production from MAIT cells purified from the PBMC of HC. In response to PMA and IM stimulation, MAIT cells produced substantial levels of IFN-γ and IL-17 (Fig. 3B) but not IL-4 or IL-10 (data not shown). However, none of these responses was observed when MAIT cells were stimulated through CD3 and CD28 (Fig. 3B). These results suggest that the activation of MAIT cells is differently regulated from that of conventional T cells. Intracellular cytokine staining also revealed that MAIT cells produced high levels of IFN-γ in response to PMA and IM (Fig. 3C, upper panels). However, interestingly, the proportion of IFN-γ^+ MAIT cells was not different between HC and MS patients (Fig. 3C, middle left panel). We could also detect intracellular IL-17 in response to PMA and IM, but the frequency was substantially lower compared with the frequency of MAIT cells positive for IFN-γ and was not different between HC and MS patients (Fig. 3C, middle right panel). In contrast to these two cytokines, the frequencies of IL-4^+ and IL-10^+ MAIT cells were lower than those of other T cells positive for these cytokines and were not different between HC and MS patients (Fig. 3C, lower panels).

To evaluate the *in vivo* status of MAIT cells, we next measured cytokine mRNA expression in MAIT cells isolated from HC or MS without additional stimulation. As shown in Fig. 3(D), expression levels of IFN-γ and IL-17 in MAIT cells were not different from control T cells, and the values were comparable between HC and MS patients.

MAIT cells suppress IFN-γ production from T cells in a cell contact-dependent manner

To address the function of MAIT cells in peripheral blood, we evaluated whether depletion of MAIT cells from PBMC might affect cytokine production from T cells. As shown in Fig. 4(A), IFN-γ production in response to PHA stimulation was increased by depletion of MAIT cells from PBMC derived from both HC and MS patients. The enhanced production of IFN-γ by MAIT cell depletion was also observed when PBMC were stimulated through CD3 or CD3 and CD28 (Supplementary Figure 2 is available at *International Immunology* Online). The enhancement of the production was specific to IFN-γ since other cytokines including IL-4, IL-10 and IL-17 were not altered by depletion of MAIT cells from PBMC (Supplementary Figure 3 is available at *International Immunology* Online). These findings suggested that MAIT cells suppress IFN-γ production from T cells in peripheral blood. This IFN-γ suppression by MAIT cells was confirmed by adding purified MAIT cells back into PBMC from which MAIT cells had been depleted (Fig. 4B).

To further elucidate the mechanism of IFN-γ suppression by MAIT cells, we first examined the involvement of suppressive cytokines such as IL-10 and TGF-β by adding their specific mAbs to the culture. MAIT cell-mediated suppression of IFN-γ production was not altered in the presence of these mAbs (Fig. 4C). We next examined whether MAIT cell-mediated IFN-γ production requires cell contact. As shown in Fig. 4(B), the IFN-γ suppression by MAIT cells could not be observed when the cell contact between MAIT cells and other cells was blocked using transwell inserts. Since we previously showed that ICOS/ICOS-L interaction is involved in the suppression of EAE (15), we next examined the effect of anti-ICOS-L mAb in this culture system. The inhibition of IFN-γ production was similar in the presence of anti-ICOS-L mAb compared with that in the presence of control immunoglobulin (Fig. 4C). We next assessed the requirement for B cells in MAIT cell suppression of IFN-γ since we have previously shown that MAIT cell suppression of EAE was dependent on the presence of B cells in this model (15). However, as shown in Fig. 4(D), B-cell depletion had no effect on MAIT cell-dependent suppression of IFN-γ production. These findings indicate that MAIT cell-mediated suppression of IFN-γ production from T cells in peripheral blood required cell contact but not IL-10, TGF-β, ICOS or B cells.

Discussion

In this study, we show that MAIT cells, which comprise a large cell population in human peripheral blood, are reduced in MS patients, especially in those with active disease. Although the precise mechanism of this reduction of MAIT cells in the peripheral blood of MS patients could not be addressed in our present study, the trafficking of MAIT cells from blood into MS lesions is a possible explanation, especially in patients with active disease and those in relapse since we previously showed that MAIT cells invade MS lesions (16). In support of this idea, we found in this

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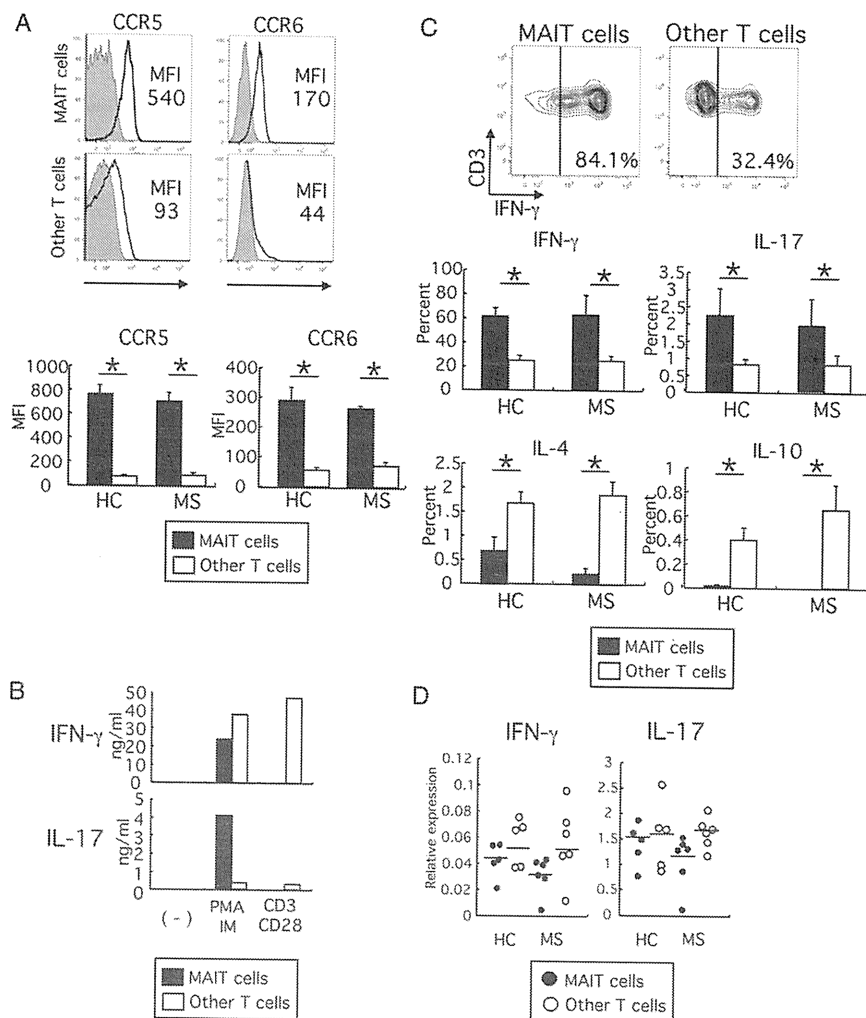

Fig. 3. Phenotype, activation properties and cytokine profile of MAIT cells. (A) Representative histograms of CCR5 and CCR6 expression on MAIT cells ($CD3^+TCR\text{-}\gamma\delta^-CD161^{high}3C10^+$) and other T cells ($CD3^+TCR\text{-}\gamma\delta^-3C10^-$) from an MS remission (upper panels). Bold lines indicate staining of the specific mAb, and shaded histograms indicate background staining of their isotype control antibodies. Mean fluorescence intensity is indicated in each histogram. Results from HC (n = 9) and MS remission (n = 6) are summarized in the lower panels. Error bars represent the SEM. *P < 0.05 (Wilcoxon t-test). (B) IFN-γ (upper panel) and IL-17 (lower panel) production from isolated MAIT cells ($CD5^+CD19^-TCR\text{-}\gamma\delta^-CD161^{high}3C10^+$) and other T cells ($CD5^+CD19^-TCR\text{-}\gamma\delta^-CD161^-3C10^-$) stimulated with PMA and IM or anti-CD3- and -CD28-mAb. Representative results from four independent experiments using cells from three HCs are shown. (C) Intracellular cytokine staining of MAIT cells ($CD3^+TCR\text{-}\gamma\delta^-CD161^{high}3C10^+$) and other T cells ($CD3^+TCR\text{-}\gamma\delta^-3C10^-$). Representative staining profiles of IFN-γ from an MS remission are shown (upper panels), and results of IFN-γ, IL-17, IL-4 and IL-10 staining from HC (n = 8) and MS remission (n = 5) are summarized (lower panels). Error bars represent the SEM. *P < 0.05 (Wilcoxon t-test). (D) IFN-γ (left) and IL-17 (right) mRNA expression in MAIT cells ($CD3^+TCR\text{-}\gamma\delta^-CD161^{high}3C10^+$) and other T cells ($CD3^+TCR\text{-}\gamma\delta^-3C10^-$) isolated from HC ($n$ = 5) and MS remission (n = 6). Each symbol represents the value of one individual. Horizontal bars indicate the means.

study that MAIT cells express high levels of CCR5, CCR6 and α4β1 integrin (data not shown), molecules that are important in the infiltration of T cells into MS lesions, although expression level of these molecules were not different between HC and MS patients. In addition to these findings in MS, it was recently shown that MAIT cells express specific pattern of chemokine receptor (14) and infiltrate lesions resulting from bacterial infection (19, 20), chronic inflammatory demyelinating polyneuropathy (16) and kidney and brain tumors (22). These findings suggest that it is the MAIT cells' character to infiltrate inflammatory lesions.

A second possible explanation for the reduced MAIT cell frequency in the PBMC of MS patients is developmental impairment of MAIT cells in patients. It was previously shown that the development and peripheral expansion of MAIT cells were dependent on the host's microbiological environment (12, 13). In addition, recent epidemiological studies pointed out a universal increase in the prevalence of MS over time (23) and emphasized the importance of changes in environmental factors including sanitation and food quality, factors that affect the profile of intestinal microflora. In this context, our hypothesis is that the change in sanitation status and

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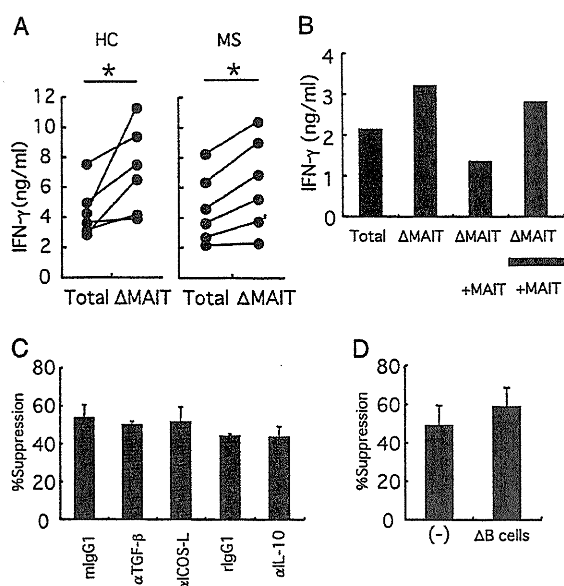

Fig. 4. Suppression of IFN-γ production from T cells by MAIT cells. (A) IFN-γ production from PBMC stimulated with PHA with (ΔMAIT) or without (total) depletion of MAIT cells in HC and MS remission (n = 6 each). $^*P < 0.05$ (Wilcoxon t-test). (B) IFN-γ production from MAIT cell-depleted PBMC (ΔMAIT) with (+MAIT) or without addition of MAIT cells. Horizontal bar indicates the presence of transwell inserts between MAIT cells and other cells. A representative result from four independent experiments using PBMC from three HCs is shown. (C and D) Efficiency of IFN-γ suppression (%suppression) by MAIT cells in the presence of mAbs against TGF-β, ICOS-L, IL-10 (C) or when B cells were depleted from the culture (ΔB cells) (D). Mean ± SEM of three independent experiments using PBMC from three HCs are shown.

quality of food intake has induced alterations in the profile of gut microflora and impaired the development of MAIT cells and, consequently, resulted in the increased prevalence of MS. On the other hand, genetic factors may also be relevant to an impairment in MAIT cell development in MS. In this regard, it is interesting to note that a single-nucleotide polymorphism in the CD161 molecule, which is expressed at high levels in MAIT cells, has been correlated with MS susceptibility (24); however, the role of this molecule in MAIT cell function and development has not been elucidated.

We observed positive correlations between the frequency of MAIT cells and those of CD4$^+$iNKT and CD56^{bright} NK cells in HC. In contrast, these correlations were not observed in MS patients. These findings suggest the existence of an immune-regulatory link among these innate lymphocyte populations wherein they cooperate to regulate autoimmune responses and imply that the immune pathology of MS is related to a disruption in this regulatory link. In addition, these findings suggest that studies on the immune pathology of MS should not focus only on a single cell population but should also take notice of the system within which the immune cells exist. On the other hand, we cannot deny that these cell populations in peripheral blood are regulated independently. Indeed, while MAIT cells have a propensity to infiltrate inflammatory tissues, CD56^{bright} NK cells are known to migrate into lymph nodes.

An interesting property of human MAIT cells identified in this study is their non-responsiveness to CD3 and CD28 stimulation *in vitro*. This is consistent with a previous report that the CD8$^+$CD161^{high} T-cell population was not responsive to CD3 and CD28 stimulation even in the presence of exogenous IL-2 (25). We confirmed that most of this CD8$^+$CD161^{high}T cells express Vα7.2 TCR and correspond to MAIT cells. On the other hand, it was recently shown that MAIT cells respond to antigen-presenting cells by producing IFN-γ in an MR1-dependent manner only when the antigen-presenting cells are infected with bacteria (20). These observations suggest a unique activation property of MAIT cells, although the precise mechanism of activation and the cognate antigen are unknown.

We demonstrated in this study that MAIT cells suppress IFN-γ production from T cells and suggest a disease-suppressive role for MAIT cells in MS via suppression of autoreactive T_h1 cells. A suppressive role for MAIT cells was similarly seen in the mouse EAE model and preferential suppression of IFN-γ over other cytokines was also observed in this system (15). With regard to the mechanism of suppression, however, factors other than the requirement for cell contact were quite different between these two species. In mice, the interaction of MAIT cells with B cells through ICOS induced IL-10 production from both cell populations, and this IL-10 up-regulation was associated with EAE amelioration (15). In contrast, we could not detect ICOS expression (data not shown) or IL-10 production by human MAIT cells and suppression of IFN-γ did not require IL-10, ICOS or B cells. In addition, other MAIT cell differences between these species have been reported: human MAIT cells express zinc finger-and BTB domain-containing protein 16 (ZBTB16) transcription factor (26), show a memory phenotype (13, 14), are anergic to CD3 and CD28 stimulation and produce mainly pro-inflammatory cytokines in response to PMA and IM. In contrast, mouse MAIT cells are negative for ZBTB16 (13), show a naive phenotype (13) and respond to TCR stimulation by producing both pro- and anti-inflammatory cytokines (27, 28). The reason for these differences is not clear, but one possibility is the difference in commensal flora that these species are exposed to during their evolution.

In contrast to the present findings, a pro-inflammatory role for MAIT cells in MS cannot formally be ruled out since MAIT cells produced IFN-γ and IL-17 in response to PMA and IM stimulation in this study. Similar finding was reported recently by Dusseaux *et al.* (14). However, similar to us, they could not detect IL-17 production in response to CD3 and CD28 stimulation even in the presence of IL-18 or IL-23, in contrast to the high level of IL-17 and IFN-γ production from MAIT cells stimulated with PMA and IM. Therefore, as the activation properties of MAIT cells are quite unique and as the signal(s) required for MAIT cell activation in MS is largely unknown, conclusions from studies using only PMA and IM do not necessarily reflect the *in vivo* cytokine profile of this cell population. This question requires further studies analyzing the cytokine profile of MAIT cells in MS lesions without exogenous stimulation. In this regard, the results of our cytokine mRNA quantification in unstimulated MAIT cells from peripheral blood are in contradiction to the inflammatory nature of this cell population in MS.

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In summary, we show that MAIT cells are reduced in the peripheral blood of MS patients and that their frequency reflects the disease activity of MS. Moreover, we found that MAIT cells, consistent with an immune-regulatory link with other innate immune cell populations, provide a disease-suppressive role in MS by repressing IFN-γ production from T cells. We hypothesize that MAIT cells act as a sensor for environmental changes by responding to alterations in gut microflora by modulating the host's immune system. This property of MAIT cells should be favorable for host defense in most cases but may be disadvantageous in some case including MS. It is possible, however, that a novel treatment for MS might be established by enhancing the immunosuppressive property of MAIT cells through modulation of the host's gut microflora.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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GRAIL (Gene Related to Anergy in Lymphocytes) Regulates Cytoskeletal Reorganization through Ubiquitination and Degradation of Arp2/3 Subunit 5 and Coronin 1A*[S]

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Anergy is an important mechanism for the maintenance of peripheral tolerance and avoidance of autoimmunity. The up-regulation of E3 ubiqitin ligases, including GRAIL (gene related to anergy in lymphocytes), is a key event in the induction and preservation of anergy in T cells. However, the mechanisms of GRAIL-mediated anergy induction are still not completely understood. We examined which proteins serve as substrates for GRAIL in anergic T cells. Arp2/3-5 (actin-related protein 2/3 subunit 5) and coronin 1A were polyubiquitinated by GRAIL via Lys-48 and Lys-63 linkages. In anergic T cells and GRAIL-overexpressed T cells, the expression of Arp2/3-5 and coronin 1A was reduced. Furthermore, we demonstrated that GRAIL impaired lamellipodium formation and reduced the accumulation of F-actin at the immunological synapse. GRAIL functions via the ubiquitination and degradation of actin cytoskeleton-associated proteins, in particular Arp2/3-5 and coronin 1A. These data reveal that GRAIL regulates proteins involved in the actin cytoskeletal organization, thereby maintaining the unresponsive state of anergic T cells.

The regulation of T cell activation ensures efficient elimination of pathogens, as well as the maintenance of tolerance to self. Peripheral tolerance prevents the expansion of self-reactive T cells that escaped thymic selection, thus avoiding autoimmunity. T cell anergy is one form of peripheral tolerance that results in nonresponsiveness to antigen rechallenge following an initial partial activation; partial initial activation may result from the stimulation of T cell receptor (TCR)[2] in the absence of co-stimulation or the stimulation of T cells with the calcium ionophore ionomycin (1, 2). The induction of T cell anergy is inhibited by the addition of cyclohexamide, suggesting that anergy induction requires new protein synthesis (3). Recent reports have demonstrated that the induction of E3 ubiquitin ligases, including CBL-b, Itch, Deltex-1, and GRAIL (gene related to anergy in lymphocytes), is required to induce and maintain T cell anergy (4–8). In particular, it is well known that Cbl and Cbl-b act as negative regulators of TCR or CD28 signal transduction cascade through their ability to ubiquitinate tyrosine kinases including Src family kinases such as Fyn and Lck; Syk family kinases such as ZAP-70, Syk, PKC-θ, phospholipase C-γ, and p85; and the regulatory subunit of PI3K (4, 5, 9–15).

GRAIL is a type I transmembrane E3 ligase identified as an early gene that promotes T cell anergy (8). The up-regulation of GRAIL was observed in anergic CD4 T cells after treatment with ionomycin *in vitro* (4). Overexpression of GRAIL in T cell hybridomas or in primary cells reduces IL-2 production as well as proliferation upon antigen stimulation. Naive T cells from *GRAIL*-deficient mice exhibit increased proliferation and cytokine expression upon activation compared with those from control mice and do not depend on co-stimulation for effector generation (16, 17). Moreover, *GRAIL*-deficient mice exhibit lymphocyte infiltration into the lung and kidney and exacerbation of experimental autoimmune encephalomyelitis, indicating an important role for GRAIL in preventing lymphoproliferative and autoimmune responses (17). Although several candidates for GRAIL targets have been reported, including membrane proteins such as CD40 ligand and cytosolic proteins such as Rho GDIs, the mechanisms of GRAIL-mediated anergy induction are still not completely understood (18–21).

T cell activation and function require a structured engagement of antigen-presenting cells. These cell contacts are characterized by prolonged contacts from stable junctions called immunological synapses (IS). Reorganization of the actin cytoskeleton plays an important role in IS formation and signaling. Treatment of T cells with the actin-destabilizing agent cytochalasin D inhibits TCR-mediated IL-2 gene transcription (22). The Arp2/3 (actin-related protein 2/3) complex has been reported to be essential for TCR-mediated cytoskeletal reorganization (23, 24), and Arp2/3 complex-mediated actin nucleation is required for the formation of an F-actin-rich lamellipod (22, 25, 26). Coronin 1A is preferentially expressed in hematopoietic cells and co-localizes with F-actin-rich membranes in activated T cells (27). Coronin 1A has been shown to bind the Arp2/3 complex and inhibit F-actin nucleation by freezing the Arp2/3 complex in its inactive conformation (28). *Coronin 1A*-deficient T cells exhibit reduced cytokine production, including of IL-2 and IFN-γ, and altered F-actin reorganization (29). Moreover, a nonsense mutation in coronin 1A was identified as a gene alteration associated with the Lmb3 locus, which

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental text, Table S1, and Figs. S1 and S2.
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[2] The abbreviations used are: TCR, T cell receptor; IS, immunological synapse(s); OVA, ovalbumin; Ab, antibody; Ub, ubiquitin.

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plays a major role in modulating autoimmunity in Fas^{lpr} mice (30).

In the present study, we demonstrate that both Arp2/3 subunit 5 (Arp2/3-5), a component of the Arp2/3 complex, and coronin 1A serve as substrates for GRAIL. The expression of Arp2/3-5 and coronin 1A is reduced in anergic T cells and in T cells in which GRAIL is overexpressed. Retroviral-driven expression of Arp2/3-5 or coronin 1A in anergic ovalbumin (OVA)-specific T cells restores their proliferation upon antigen activation. The accumulation of F-actin, Arp2/3-5, and coronin 1A at the IS is decreased in anergic T cells as well as in T cells overexpressing GRAIL. Thus, our findings demonstrate that GRAIL maintains the anergic states of T cells by regulating IS formation via degradation of the actin cytoskeleton-associated proteins Arp2/3-5 and coronin 1A.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—We obtained ionomycin, polybrene, and OVA from Sigma-Aldrich, OVA peptide ($OVA_{323-339}$) from TORAY Laboratory (Tokyo), lactacystin from Boston Biochem Inc., and recombinant IL-2 from Pepro Tech. We purchased antibodies (Abs) against Arp2/3-5 (C3), c-Myc (9E10), HA (F7), and GAPDH (6C5) from Santa Cruz Biotechnology, anti-Arp2/3-5 from Epitomics Inc. (Burlingame, CA), anti-coronin 1A from Everest Biotech Ltd. (Oxfordshire, UK), anti-CD28 Ab from BD Bioscience (San Jose, CA), and peroxidase-conjugated anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG from DAKO-Japan (Tokyo). We obtained the pcDNA4-V5/His vector, pcDNA4-Myc/His vector, and SNARF-1 from Invitrogen and the pAcGFP1-N1 vector from Clontech Laboratories, Inc. HA-conjugated wild-type or mutated ubiquitin constructs were kind gifts from Dr. C. Akazawa at Tokyo Medical and Dental University. pAlter-MAX HA-Cbl-b was a kind gift from Dr. H. Band (University of Nebraska Medical Center).

Mice—DO11.10, OVA-specific TCR-transgenic mice were purchased from Jackson Laboratories. Seven-week-old female C57BL/6J mice were purchased from CLEA Laboratory Animal Corporation (Tokyo, Japan). The animals were maintained in specific pathogen-free conditions, and all care and use procedures were in accordance with institutional guidelines.

Cell Culture and Proliferation—DO11.10 splenocytes were cultured in complete DMEM (Invitrogen) supplemented with 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin/streptomycin, and 10% FBS. Proliferative responses after 2 days of stimulation with plate-bound anti-CD3 (0.5 μg/ml) and anti-CD28 (1 μg/ml) Abs were determined by [^{3}H]thymidine incorporation using a β-1205 counter (Pharmacia). To induce anergy *in vitro*, DO11.10 splenocytes incubated with 1 mg/ml OVA for 3 days were rested for 7–10 days and were then stimulated for 18 h with ionomycin (1 μg/ml) (3).

Constructs—GRAIL, Arp2/3-5, coronin 1A, RhoGDIα, RhoGDIβ, Lasp1, and RGS10 cDNAs from DO11.10 T cells in which anergy had been induced by ionomycin were amplified with following the specific PCR primers: GRAIL, 5′-CAGTGAATTCATGGGGCCGCCGCCCGGGATC-3′ and 5′-CAGTCTCGAGAGATTTAATCTCCCGAACAGCAGC-3′; Arp2/3-5, 5′-CATGGAATTCTCCGGGATGTCGAAGAACACGGTGTC-3′ and 5′-GATCGCGGCCGCCACGGTTTTCCTTGCAGTCA-3′; coronin1A, 5′-GATCGCGGCCGCCTACTTGGCCTGAACAGTCT-3′ and 5′-CAGTCTCGAGCTTGGCCTGAACAGTCTCCTC-3′; RhoGDIα, 5′-CATGGAATTCGTAAGCATGGCAGAACAGGAACCCAC-3′ and 5′-GATCGCGGCCGCGTCCTTCCACTCCTTTTTGA-3′; RhoGDIβ, 5′-CATGGGATCCATCAAGATGACGGAGAAGGATGCACA-3′ and 5′-GATCGCGGCCGTTCTGTCCAATCCTTCTTAA-3′; and RGS10, 5′-CAGTGGATCCATGTTCACCCGCGCCGTG-3′ and 5′-CAGTCTCGAGTGTGTTGTAAATTCTGGAGGCTCG-3′. SOD1 cDNA from brain was amplified with the following PCR primers: 5′-CAGTGAATTCATGGCGATGAAAGCGGTGTGC-3′ and 5′-CAGTCTCGAGCTGCGCAATCCCAATCACTCC-3′. PCR products were cloned into a pcDNA4 V5/His vector or pcDNA4 Myc/His vector. The H297N and H300N mutations in the RING domain of murine GRAIL were generated using a PCR site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Deletion of the RING domain in murine GRAIL was generated using the following PCR primers: for the 5′-PCR product, CAGTGAATTCATGGGGCCGCCGCCCGGGATC and CAGTTTCGAATCTCCATCAGGGCCAATTTC; and for the 3′-PCR product, CAGTTTCGAAGTGTGACATTCTCAAAGCT and CAGTCTCGAGAGATTTAATCTCCCGAACAGCAGC. After these reactions, the DNAs were digested with BamHI and HpaI, and the fragments, which were WT-GRAIL-V5/His, H2N2-GRAIL-V5/His, ΔRF-GRAIL-V5/His, Arp2/3-5-Myc/His, coronin 1A-Myc/His, RhoGDIα-Myc/His or RhoGDIβ-Myc/His, were subcloned into a pMIG vector. After pcDNA4 WT-GRAIL-V5/His was digested with NheI and XhoI, the fragment was subcloned into pAcGFP N1 vector.

Retroviral Transductions and Proliferation of Transfected T Cells—HEK293T cells were transfected with a pMIG plasmid and pCLEco helper plasmid by calcium phosphate precipitation. Supernatants were collected 48 and 72 h later and filtered through 0.45-μm syringe filters (Millipore, MA). Activated DO11.10 $CD4^+$T cells were resuspended in the collected supernatant (1×10^6 cells/ml) with recombinant IL-2 (50 units/ml) and polybrene (8 μg/ml) and were centrifuged at 2,500 rpm for 90 min. Transfected cells were expanded in complete DMEM with recombinant IL-2 for 48 h and were rested without IL-2. After treatment with ionomycin (0.3 μg/ml) for 18 h, the cells were stained with SNARF-1 (5 μM) for 15 min and were stimulated with plate-bound anti-CD3 and anti-CD28 Abs. Two days later, proliferation was analyzed using a FACSCalibur and the CELLQuest program (BD Biosciences).

Western Blot Analysis—The cells were washed with PBS and lysed in 1% Nonidet P-40 lysis buffer (137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 20 mM Tris, pH 7.5). After incubation for 10 min on ice, lysates were centrifuged at 13,200 rpm for 15 min at 4 °C, and supernatants were collected. After adjustment of protein concentrations using the Dc protein assay (Bio-Rad), the lysates were mixed with Laemmli's buffer (1.33% SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.002% bromphenol blue, 83 mM Tris, pH 6.8) and were boiled for 5 min. Lysates (10–30 μg) were subjected to 10 or 12% SDS-PAGE and immobilized on nitrocellulose membranes. The membranes were blocked with 5% milk, PBS, 0.05% Tween for 1 h at room temperature. Proteins were detected with various Abs (mostly diluted at 1:1000)

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and horseradish peroxidase-coupled anti-rabbit, anti-mouse, or anti-goat IgG Abs (1:1000). The proteins were visualized using an enhanced chemiluminescence Western blot detection system (Amersham Biosciences).

Ubiquitination Assay—HEK293T cells were co-transfected with V5/His-tagged GRAIL, HA-tagged ubiquitin, and Myc/His-tagged substrate-containing expression vectors. Twenty-four hours later, the cells were incubated with 0.3 μM lactacystin for 12 h. The cells were lysed using 1% Nonidet P-40 lysis buffer containing protease inhibitors (Complete protease inhibitor mixture; Roche Applied Science) and were subjected to immunoprecipitation with anti-Myc Ab. Ubiquitination of substrates was analyzed by SDS-PAGE after blotting with anti-HA Ab.

Immunofluorescence Microscopy—To investigate co-localization of GRAIL and its substrates, HEK293T cells were co-transfected by calcium phosphate precipitation with the pAcGFP1-N1 vector containing GRAIL and pcDNA4-DsRed vector containing the substrate. Twenty-four hours later, the cells were incubated with lactacystin (0.3 μM) for 12 h and were fixed with MeOH for 15 min at 4 °C. To analyze T cell-B cell conjugation, A20 cells pulsed with 1 μg/ml $OVA_{323-339}$ for 2 h at 37 °C were incubated at a ratio of 1:1 with transfected GFP^+DO11.10 $CD4^+$T cells sorted on a FACS Aria cell sorter (BD Biosciences) at 37 °C for 10 min. The cells were then plated on poly-L-lysine-coated slides for 15 min. To analyze lamellipodium formation, T cells overexpressing the control or indicated constructs were settled onto anti-CD3-coated coverslips for 5 min as described previously (26). The cells were fixed with 4% paraformaldehyde for 15 min at 4 °C and washed with PBS, 0.01% Tween 20. After blocking with PBS, 1% BSA for 1 h at room temperature, the cells were incubated with either anti-Arp2/3-5 (C3) or anti-coronin 1A Ab for 18 h at 4 °C. After washing, the cells were labeled with Cy5-conjugated anti-mouse IgG or anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. The slides were mounted with ProLong Gold antifade reagent (Invitrogen) with or without DAPI. Confocal images were acquired using FV1000-D (Olympus, Tokyo, Japan).

Statistical Analysis—Statistical differences between control and treatment groups were assessed with the Student's *t* test.

Additional Procedures—Information on semiquantitative RT-PCR and generation of shRNA is available in the supplemental materials.

RESULTS

Reduced Expression of Arp2/3-5 and Coronin 1A—E3 ubiquitin ligases including GRAIL are up-regulated in anergized T cells and play an important role in the induction of anergy (4, 8). To determine which proteins serve as substrates for GRAIL, we used two-dimensional difference gel electrophoresis to analyze proteins that were down-regulated in T cells in which anergy had been induced by ionomycin. Down-regulated proteins were identified by MALDI-TOF-MS and the nonredundant NCBI (NCBInr) database using MASCOT software (supplemental Table S1). Proteins related to cytoskeletal reorganization were the most frequently down-regulated proteins in anergic T cells. We decided to focus on actin-related proteins Arp2/3-5 and coronin 1A. We first confirmed that the expression levels of these proteins were reduced in T cells in ionomycin-induced anergy. We stimulated splenocytes of DO11.10 mice with OVA protein for 3 days and then rested them for 7 days. Anergy was induced by the addition of ionomycin for 18 h and the proliferative response upon the addition of anti-CD3 and anti-CD28 Abs detected by the incorporation of [^{3}H]thymidine. The proliferative response was significantly suppressed in ionomycin-treated cells, confirming that anergy was properly induced (Fig. 1*A*). In these anergized cells, the protein expression of Arp2/3-5 and coronin 1A was reduced (Fig. 1*B*, *lanes 2* and *4*). To address the functional involvement of Arp2/3-5 and coronin 1A in T cell anergy, we examined whether overexpression of these proteins in DO11.10 $CD4^+$ T cells enhanced their proliferative response upon stimulation. DO11.10 $CD4^+$ T cells were transfected with Arp2/3-5 or coronin 1A. To analyze proliferation of transfected T cells by flow cytometry, the cells were treated with ionomycin and labeled with SNARF-1, which can monitor proliferating cells through dye dilution in a similar fashion to CFSE dilution assay. The number of proliferating cells upon stimulation (GFP^+ SNARF-1^- cells) was increased in Arp2/3-5 or coronin 1A-overexpressing cells compared with that of control cells (Fig. 1*C*). We also analyzed whether an anergy-like state was displayed by knockdown of Arp2/3-5 or coronin 1A. The percentage of proliferation increase upon the restimulation with anti-CD3/anti-CD28 was decreased in Arp2/3-5 shRNA-expressing T cells (8%) and in coronin 1A shRNA-expressing T cells (3%) compared with that in control shRNA-expressing cells (13%). These results indicate that the expression of Arp2/3-5 and coronin 1A is correlated with T cell responses and is reduced in anergic T cells.

GRAIL Polyubiquitinates Arp2/3-5 and Coronin 1A—We next examined whether Arp2/3-5 and coronin 1A serve as substrates for GRAIL. Myc-tagged Arp2/3-5, coronin 1A or other candidate substrate proteins were transiently co-expressed with V5-tagged GRAIL and HA-tagged ubiquitin (Ub) in HEK293T cells. Twenty-four hours after transfection, the cells were treated with the proteasome inhibitor lactacystin for 12 h, and then lysates were prepared and immunoprecipitated with an anti-Myc Ab. SDS-PAGE followed by immunoblotting with anti-HA revealed a polyubiquitinated laddering pattern of Arp2/3-5 and coronin 1A in the presence of GRAIL (Fig. 2*A*, *lanes 6* and *10*). As Rho GDP dissociation inhibitors (RhoGDI) α and β were previously reported as substrates of GRAIL, we confirmed that these two proteins were polyubiquitinated as well (Fig. 2*A*, *lanes 8* and *4*). On the other hand, Lasp1 (LIM and SH3 protein 1), RGS10 (regulator of G-protein signaling 10), and SOD1 (superoxide dismutase 1), which were identified as proteins with reduced expression in anergized T cells by the two-dimensional difference gel electrophoresis analysis, were not ubiquitinated in the presence of GRAIL (Fig. 2*A*, *lanes 2*, *12*, and *14*). These results indicate that Arp2/3-5 and coronin 1A are selectively polyubiquitinated by GRAIL. Histidine to asparagine substitution in the RING finger domain (H2N2) or deletion of the RING finger domain (ΔRF) of GRAIL (Fig. 2*B*) reportedly inactivates GRAIL. These mutant forms of GRAIL abrogated the ability of GRAIL to ubiquitinate Arp2/3-5 and coronin 1A as well as RhoGDIα and β (Fig. 2*C*). Recent evi-

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