morphologically indistinguishable from the CD138$^-$ population in NMO patients or HS, indicating the immature characteristic of CD27^{high}CD38^{high}CD180$^-$ cells (Fig. S6). These phenotypical and morphological characteristics as well as the results of the quantitative real-time PCR (qRT-PCR) analysis indicate that this B-cell population is equivalent to plasmablasts (PB) (22–26). Hereafter, we use the term "PB" to distinguish this population from other B cells.

Expression of B-Cell Cytokine Receptors on PB. Prior studies have identified cytokines that are critically involved in the differentiation and/or survival of plasma cells, including IL-6 and B-cell–activating factor (BAFF). IL-6 induces B-cell differentiation into plasma cells, maintains early plasma cell survival, and enhances plasma cell IgG secretion (24). Besides, IL-6 is elevated in the cerebrospinal fluid (CSF) or peripheral blood of NMO patients compared with that of CMS patients and HS (27, 28). In a rodent autoimmunity model, IL-6 deficiency caused impaired autoantibody secretion by B cells (29). Given the potential role of IL-6 in NMO, we performed flow cytometry analysis for the expression of IL-6R. Results showed remarkable expression of IL-6R on PB, although it was only marginal or absent on mB and nB (Fig. S7). Because BAFF and A proliferation-inducing ligand (APRIL) can also promote the survival of PB (25, 26), we next evaluated the expression of the receptors for BAFF and APRIL, BAFF receptor (BAFF-R), B-cell maturation antigen (BCMA), and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI). Expression of BCMA and TACI was selectively up-regulated in PB in parallel with IL-6R. In contrast, BAFF-R was up-regulated in mB and nB, but not in PB (Fig. S7).

PB Is a Selective Source of AQP4-Abs in Peripheral Blood. We were interested to know whether PB were capable of producing AQP4-Abs upon stimulation with cytokines and, therefore, examined the ability of IL-6, BAFF, and APRIL to enhance AQP4-Ab secretion by PB. We cultured the isolated PB for 6 d in the presence or absence of each cytokine, and evaluated the presence of AQP4-Abs in the supernatants by measuring IgG binding to Chinese hamster ovary (CHO) cells transfected with the human AQP4 vector (CHO^{AQP4}) or the vector control (CHO^{VC}). We found that IL-6, but not BAFF or APRIL, could significantly enhance AQP4-Ab secretion from PB (Fig. S8), as assessed by specific IgG binding to CHO^{AQP4}. Further study focusing on IL-6 showed that exogenous IL-6 promoted the production of AQP4-specific IgGs from PB (Fig. 3*A*), but not from the other B-cell subpopulations. Similar results were obtained from six independent experiments (Fig. S9), indicating that PB could be major AQP4-Ab producers in PBMC. In the absence of addition of IL-6, supernatants from PB did not show any significant reactivity to CHO^{AQP4}. To further analyze the AQP4-Ab–secreting potential of each B-cell subpopulation, we next stimulated the cells with a combination of IL-6, IL-21, and anti-CD40 that efficiently induces B-cell differentiation and IgG production (30). This polyclonal stimulation induced the secretion of similar amounts of IgGs from mB and PB. However, only the supernatant of PB specifically reacted to CHO^{AQP4} cell transfectants, indicating that AQP4-Ab–producing B cells were highly enriched in PB (Fig. 3*B*).

Survival and Functions of PB Depend on IL-6 Signaling. We evaluated the influence of IL-6, BAFF, and APRIL on the survival of PB after 2 d of in vitro culture (Fig. 4*A*). Among the added cytokines, only IL-6 was found to significantly promote the survival of PB (Fig. 4*B*). We also assessed the expression levels of X-box-binding protein 1 (XBP-1) in PB by qRT-PCR after 24 h of culture with or without IL-6. XBP-1 is a transcription factor critical for IgG secretion (31), and the splicing process of XBP-1 mRNA yields a more active and stable protein. We found that the expression of both unspliced [XBP-1(u)] and spliced [XBP-1(s)] forms of XBP-1 mRNA was augmented in PB by the ad-

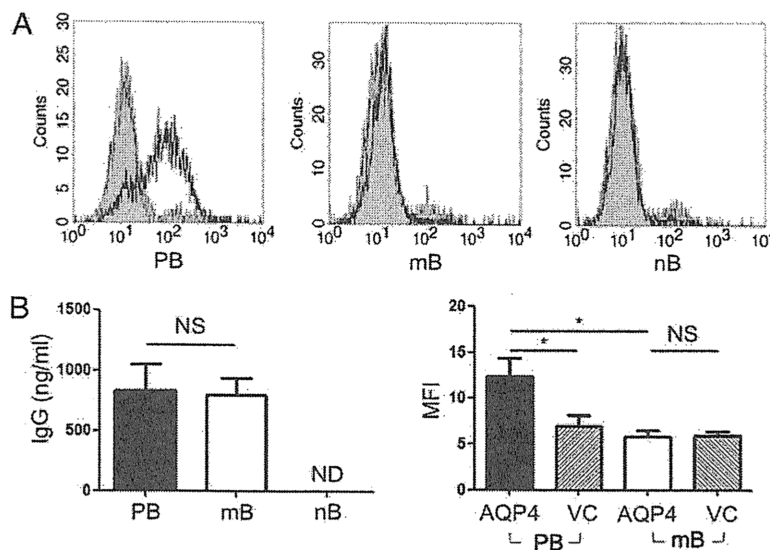

Fig. 3. Production of AQP4-Abs by PB. (*A*) Using flow cytometry, we examined whether AQP4-Abs could be produced by PB, mB, or nB cells. FACS-sorted cells were cultured with IL-6 (1 ng/mL) for 6 d and supernatants were collected. Supernatant IgGs reactive to CHO^{AQP4} (open histogram) and CHO^{VC} cells (closed histogram) were detected by anti-human IgG secondary antibody. The supernatant from PB (*Left*), but not from mB or nB, contains IgGs reactive to CHO^{AQP4}, indicating that only PB secrete AQP4-Abs after stimulation with IL-6. (*B*) Memory B cells (mB) produce IgGs but not AQP4-Abs. B-cell subpopulations were cultured in the presence of IL-6 (1 ng/mL), IL-21 (50 ng/mL), and anti-CD40 mAb (1 μg/mL) for 6 d. IgGs in the culture supernatants were measured by sandwich ELISA (*Left*) (each assay was performed in quadruplicate). Data from three patients are expressed as mean ± SD. The activity of AQP4-Abs in the culture supernatants from PB and mB was also measured by flow cytometry (*Right*). Aliquots of CHO^{AQP4} cells (AQP4) and CHO^{VC} cells (VC) ($n = 4$ for each) were stained with the supernatant of PB or mB from every patient. Data are expressed as median fluorescence intensity values from the results of three patients (*$P < 0.05$; Tukey's post hoc test). ND, not detected; NS, not significant.

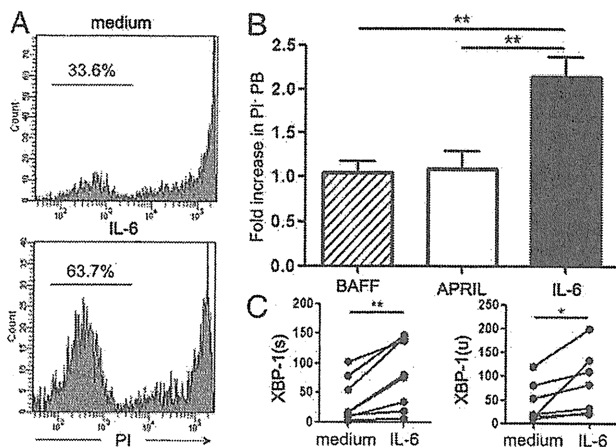

Fig. 4. Effect of exogenous IL-6 on PB. (*A*) IL-6 promotes the survival of PB. FACS-sorted PB were cultured in the presence or absence of recombinant IL-6 (1 ng/mL) for 2 d. PI staining of cultured PB showed that exogenous IL-6 increased the percentage of surviving cells (*Lower*) compared with cells 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).

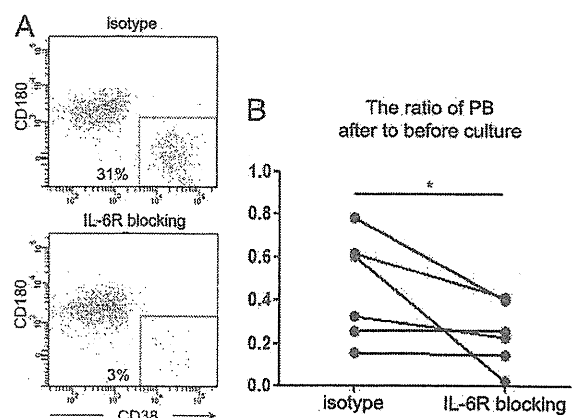

Fig. 5. IL-6R blockade selectively inhibits the survival of PB. (*A*) PBMC were cultured in a medium containing 20% autologous serum in the presence of IL-6R–blocking antibody or its isotype control mAb for 2 d. The cells were stained and analyzed as described in the experiment in Fig. 1*A*. Data represent 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.

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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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

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-$γδ^-CD161^{high}3C10^+$) or other T cells ($CD5^+CD19^-$TCR-$γδ^-CD161^-3C10^-$) 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, $CD5^+CD19^-TCRγδ^-CD161^{high}3C10^+$ 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.

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′-ACAGGGAAGCGAAAAAGGAGTCAG-3′ and IFN-γ reverse, 5′-CATGGGATCTTGCTTAGGTTGG-3′; IL-17 forward, 5′-CCAGGATGCCCAAATTCTGAGGAC-3′ and IL-17 reverse, 5′-CAAGGTGAGGTGGATCGGTTGTAG-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

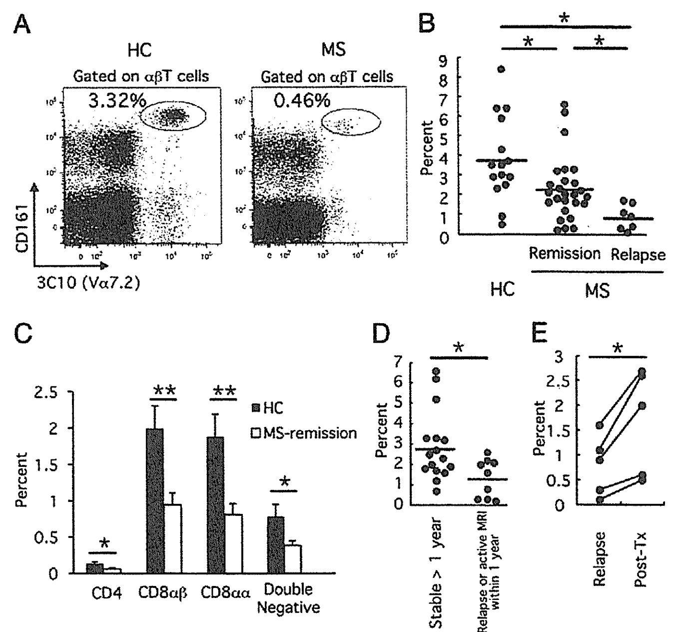

Fig. 1. Frequency of MAIT cells among αβT cells in peripheral blood. (A) Representative flow cytometry profiles of $CD3^{+}TCR\text{-}\gamma\delta^{-}$ cells in a HC (left) and in an MS relapse (right). (B) Frequency of MAIT cells among αβT cells in HC (N = 16), MS remission (N = 25) and MS relapse (N = 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).

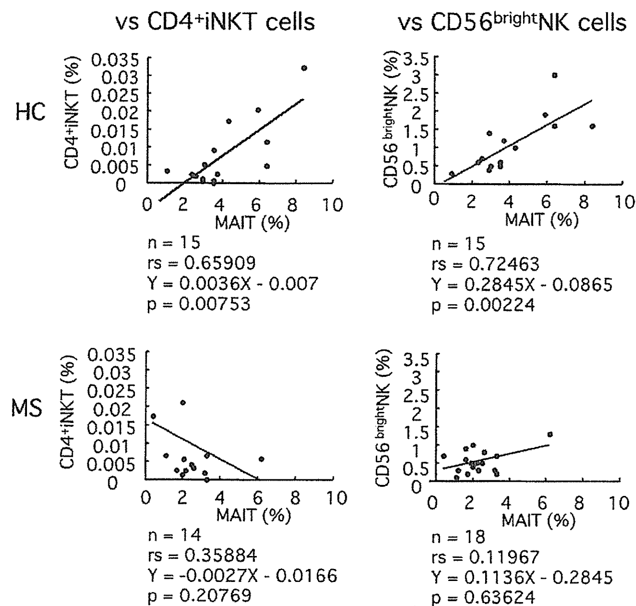

Fig. 2. Correlation of the frequency of MAIT cells with the frequency of $CD4^+$iNKT cells and $CD56^{bright}$ NK cells. The frequency of MAIT cells was plotted against the frequency of $CD4^+$iNKT cells ($CD3^+CD4^+$TCR-$V\alpha24^+$-$V\beta11^+$) among total T cells (left panels) or that of $CD56^{bright}$ 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

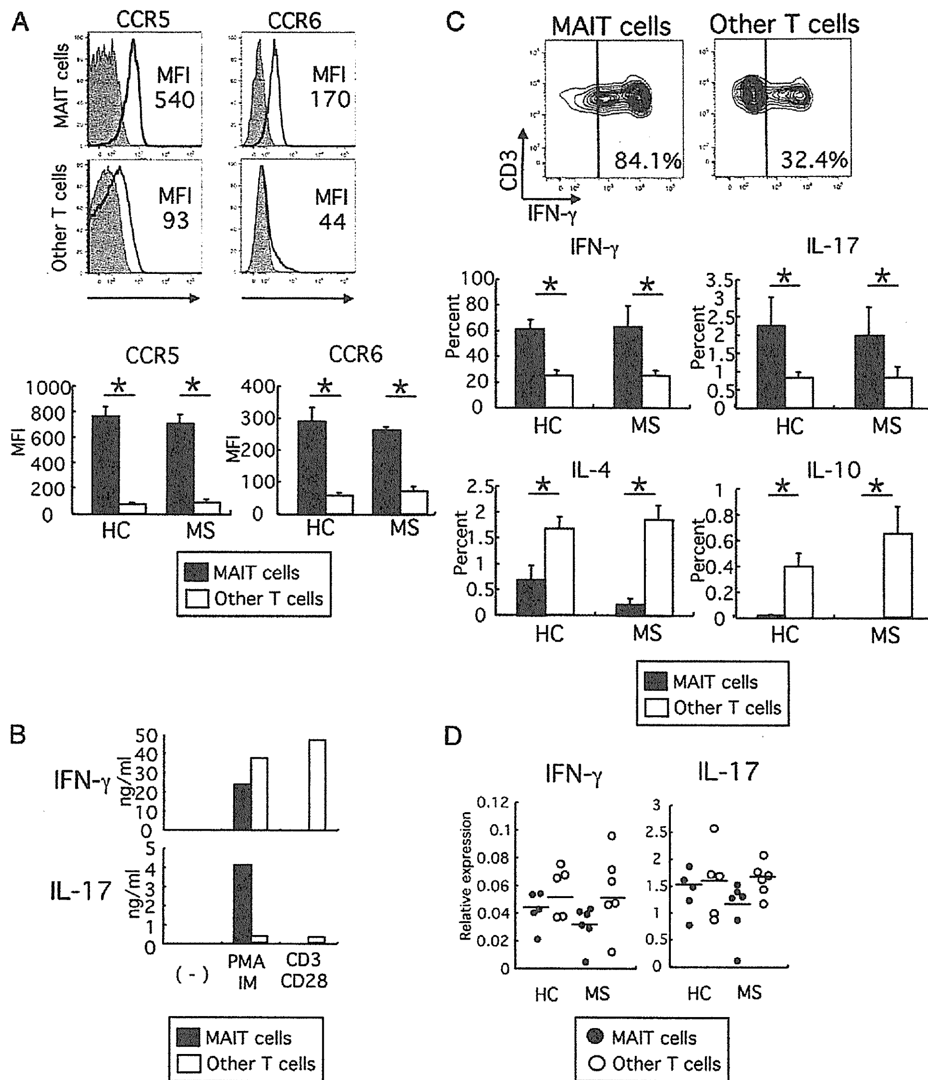

Fig. 3. Phenotype, activation properties and cytokine profile of MAIT cells. (A) Representative histograms of CCR5 and CCR6 expression on MAIT cells ($CD3^+TCR\text{-}\gamma\delta^-CD161^{high}3C10^+$) and other T cells ($CD3^+TCR\text{-}\gamma\delta^-3C10^-$) from an MS remission (upper panels). Bold 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

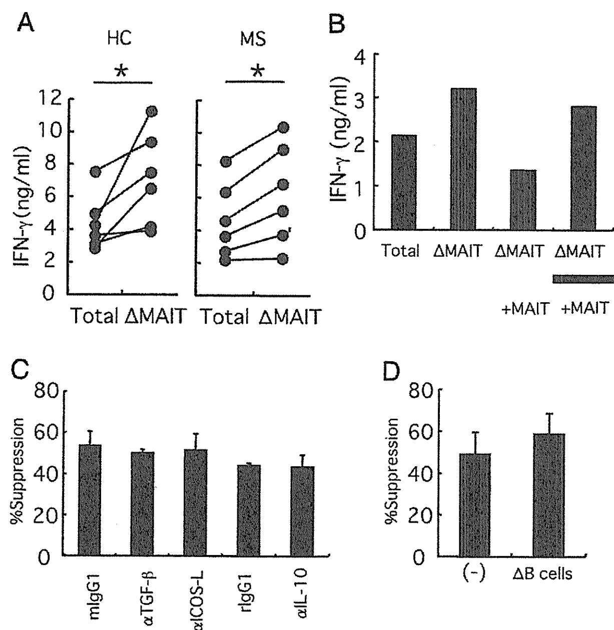

Fig. 4. Suppression of IFN-γ production from T cells by MAIT cells. (A) IFN-γ production from PBMC stimulated with PHA with (ΔMAIT) or without (total) depletion of MAIT cells in HC and MS remission ($n = 6$ each). $*P < 0.05$ (Wilcoxon *t*-test). (B) IFN-γ production from MAIT cell-depleted PBMC (ΔMAIT) 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.

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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Mucosal-Associated Invariant T Cells Promote Inflammation and Exacerbate Disease in Murine Models of Arthritis

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Objective. **The function of mucosal-associated invariant T (MAIT) cells remains largely unknown. We previously reported an immunoregulatory role of MAIT cells in an animal model of multiple sclerosis. The aim of this study was to use animal models to determine whether MAIT cells are involved in the pathogenesis of arthritis.**

Methods. **$MR1^{-/-}$ and $MR1^{+/+}$ DBA/1J mice were immunized with bovine type II collagen (CII) in complete Freund's adjuvant to trigger collagen-induced arthritis (CIA). To assess CII-specific T cell recall responses, lymph node cells from mice with CIA were challenged with CII ex vivo, and cytokine production and proliferation were evaluated. Serum levels of CII-specific antibodies were measured by enzyme-linked immunosorbent assay. Collagen antibody–induced arthritis (CAIA) was induced in $MR1^{-/-}$ and $MR1^{+/+}$ C57BL/6 mice by injection of anti-CII antibodies followed by injection of lipopolysaccharide. To demonstrate the involvement of MAIT cells in arthritis, we induced CAIA in $MR1^{-/-}$ C57BL/6 mice that had been reconstituted with adoptively transferred MAIT cells. MAIT cell activation in response to cytokine stimulation was investigated.**

Results. **The severity of CIA was reduced in $MR1^{-/-}$ DBA/1J mice. However, T and B cell responses to CII were comparable in $MR1^{-/-}$ and $MR1^{+/+}$ DBA/1J mice. $MR1^{-/-}$ C57BL/6 mice were less susceptible to CAIA, and reconstitution with MAIT cells induced severe arthritis in $MR1^{-/-}$ C57BL/6 mice, demonstrating an effector role of MAIT cells in arthritis. MAIT cells became activated upon stimulation with interleukin-23 (IL-23) or IL-1β in the absence of T cell receptor stimuli.**

Conclusion. **These results indicate that MAIT cells exacerbate arthritis by enhancing the inflammation.**

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Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in the joints. It has been suggested that environmental factors influence autoimmunity, and in particular, increasing evidence highlights the important role of gut flora in the development of autoimmune diseases (1), including arthritis. For example, differences in the intestinal microbiota of patients with early RA have been described, and tetracycline treatment was shown to reduce disease activity in RA (2,3). In addition, oral vancomycin treatment significantly decreased the severity of adjuvant-induced arthritis (4). More recently, it was demonstrated that germ-free conditions strongly inhibit arthritis in the K/BxN arthritis model and that the introduction of segmented filamentous bacteria induced severe arthritis in germ-free K/BxN mice (5). Thus, mucosal immunity plays an important role in the development and progression of arthritis.

Natural killer (NK) cells, invariant NK T (iNKT) cells, γ/δ T cells, mucosal-associated invariant T (MAIT) cells, B-1 B cells, and marginal-zone B cells are categorized as innate-like lymphocytes. Such lymphocytes reside in unique locations, including the marginal zone of the spleen and epithelial and mucosal tissues and rapidly exert effector functions in the absence of clonal expansion (6–15). Therefore, these innate-like lymphocytes are thought to play important roles in "first-line" im-

mune responses against exogenous stimuli. As MAIT cells are preferentially located in the gut lamina propria, there is a growing interest in the function of MAIT cells in various types of immune responses, including autoimmunity (16–20).

MAIT cells are restricted by a nonpolymorphic class IB major histocompatibility complex (MHC) molecule, the class I MHC–related molecule (MR1), and express an invariant T cell receptor (TCR) α-chain: $V_\alpha7.2–J_\alpha33$ in humans and $V_\alpha19–J_\alpha33$ in mice. The invariant TCRα chain associates with a limited set of V_β chains (14,21,22). MAIT cells are selected in the thymus in an MR1-dependent manner, but, interestingly, MAIT cells require B cells as well as commensal flora for their peripheral expansion (14,23). Our group previously demonstrated a protective role of MAIT cells against autoimmune encephalomyelitis (EAE), an animal model of human multiple sclerosis. The suppression of EAE was accompanied by increased production of interleukin-10 (IL-10) by B cells, which was induced in part by ICOS costimulation (17). Because the invariant $V_\alpha7.2–J_\alpha33$ TCR is highly expressed in central nervous system lesions of multiple sclerosis patients, human MAIT cells may also be involved in the pathogenesis of multiple sclerosis (16).

In addition to their regulatory function, MAIT cells also possess proinflammatory functions like other innate-like lymphocytes. Le Bourhis et al (20) demonstrated that MAIT cells display antimicrobial capacity. Both human and mouse MAIT cells are activated by *Escherichia coli*–infected antigen-presenting cells in an MR1-dependent manner. MAIT cells show a protective role against *Mycobacterium abscessus* or *E coli* infections in mice. Human MAIT cells are capable of producing interferon-γ (IFNγ) and IL-17 and are found in *Mycobacterium tuberculosis*–infected lung tissues. Thus, MAIT cells play an antimicrobial function under these infectious conditions. Although accumulating evidence suggests that certain subsets of innate-like lymphocytes, such as NK cells, iNKT cells, and γ/δ T cells, are involved in the pathogenesis of arthritis in animal models of the disease, the role of MAIT cells in arthritis remains unknown (24–31).

We report herein that MAIT cells play a pathogenic role in murine models of arthritis. The disease severity of collagen-induced arthritis (CIA) in MAIT cell–deficient $MR1^{-/-}$ DBA/1J mice was ameliorated compared with that of $MR1^{+/+}$ DBA/1J mice. However, T cell responses to type II collagen (CII) and CII-specific serum antibody levels were comparable between CIA-induced $MR1^{-/-}$ and $MR1^{+/+}$ DBA/1J mice. We found that $MR1^{-/-}$ C57BL/6J mice are much less susceptible to collagen antibody–induced arthritis (CAIA) as compared to $MR1^{+/+}$ C57BL/6J mice. $MR1^{-/-}$ C57BL/6J mice reconstituted with adoptively transferred MAIT cells developed severe arthritis, suggesting that MAIT cells may be one of the effectors contributing to inflammation in arthritis. Finally, we investigated the cytokine-producing capacity of MAIT cells. No differences in IFNγ production by liver mononuclear cells (LMNCs) from $MR1^{-/-}$ C57BL/6J and $MR1^{+/+}$ C57BL/6J mice were observed upon TCR stimulation, but the level of IL-17 produced by LMNCs from $MR1^{+/+}$ C57BL/6J mice was much higher than that produced by cells from $MR1^{-/-}$ C57BL/6J mice. We further demonstrated that sorted murine MAIT cells produce IL-17 upon TCR engagement. Surprisingly, IL-17 production by MAIT cells was observed after exposure to IL-23 without TCR stimulation, and IL-1β alone induced proliferation of MAIT cells, indicating that MAIT cells may be activated by cytokines and may enhance the inflammation in arthritis.

MATERIALS AND METHODS

Mice. DBA/1J mice were purchased from the Oriental Yeast Company. C57BL/6J mice were obtained from CLEA Laboratory Animal Corporation. $MR1^{-/-}$ mice (14) were provided by S. Gilfillan (Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO), and $V_\alpha19$i-transgenic mice (32) on a C57BL/6J background were provided by M. Shimamura (University of Tsukuba, Ibaraki, Japan). $MR1^{-/-}$ mice were backcrossed to DBA/1J mice for 10 generations to obtain $MR1^{-/-}$ DBA/1J mice. $V_\alpha19$i-transgenic $CD1d1^{-/-}$ C57BL/6J mice were generated by backcrossing $V_\alpha19$i-transgenic mice with $CD1d1^{-/-}$ C57BL/6J mice for 7 generations. Mice were maintained under specific pathogen–free conditions in accordance with institutional guidelines and used in the experiments at 7–12 weeks of age.

Induction of CIA. Both $MR1^{-/-}$ DBA/1J mice and their littermate controls ($MR1^{+/+}$ DBA/1J mice) (n = 5–6 per group; ages 7–8 weeks old) were immunized intradermally at the base of the tail with 150 μg of CII (Collagen Research Center) emulsified with an equal volume of complete Freund's adjuvant containing 250 μg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco). Three weeks after the primary immunization, mice were given an intradermal booster injection of 150 μg of CII emulsified in incomplete Freund's adjuvant (Difco).

Induction of CAIA. $MR1^{-/-}$ C57BL/6J mice and their littermate controls ($MR1^{+/+}$ C57BL/6J mice) were injected intravenously with a mixture of anti-CII monoclonal antibodies (mAb) (Arthrogen-CIA mAb, 2 mg; Chondrex) followed 2 days later by an intraperitoneal injection of 50 μg of lipopolysaccharide.

Clinical assessment of arthritis. Mice were examined for signs of joint inflammation, which was scored on a scale of 0–4, where 0 = no change, 1 = significant swelling and redness

of 1 digit, 2 = mild swelling and erythema of the limb or swelling of ≥2 digits, 3 = marked swelling and erythema of the limb, and 4 = maximal swelling and redness of the limb and later, ankylosis. The average macroscopic score was expressed as a cumulative value for all paws, with a maximum possible score of 16.

Histopathologic assessment. Arthritic mice were killed, and all 4 paws were fixed in buffered formalin, decalcified, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin. Histologic assessment of joint inflammation was scored on a scale of 0–3 as follows: 0 = normal joint, 1 = mild arthritis (minimal synovitis without cartilage/bone erosion), 2 = moderate arthritis (synovitis and erosion but joint architecture maintained), and 3 = severe arthritis (synovitis, erosion, and loss of joint integrity). The average of the macroscopic scores was expressed as a cumulative value for all paws, with a maximum possible score of 12.

CII-specific T cell response. Lymph node cells were collected on days 35–42 after immunization and suspended in complete RPMI 1640 medium (Life Technologies) containing 1% syngeneic mouse serum. The cells were cultured for 72 hours in 96-well flat-bottomed plates at a density of 1×10^6/well in the presence of CII. Proliferative responses were measured using a β-1205 counter (Pharmacia) to detect the incorporation of ^{3}H-thymidine (1 μCi/well) during the final 16 hours of culture.

Measurement of CII-specific total IgG, IgG1, and IgG2a. Bovine CII (1 mg/ml) was coated onto enzyme-linked immunosorbent assay (ELISA) plates (Sumitomo Bakelite) overnight at 4°C. After blocking with 1% bovine serum albumin in PBS, serially diluted serum samples were added to CII-coated wells. For detection of anti-CII antibodies, the plates were incubated with biotin-labeled anti-IgG1 and anti-IgG2a (SouthernBiotech) or anti-IgG antibody (CN/Cappel) for 1 hour and were then incubated with streptavidin–peroxidase. After adding substrate, the reaction was evaluated as the optical density values at 450 nm (OD_{450}).

Adoptive transfer and in vitro stimulation of $V_\alpha 19i$ T cells. LMNCs were purified from V_α19i-transgenic CD1d1$^{-/-}$ C57BL/6J mice by use of Percoll density-gradient centrifugation, and erythrocytes and B cells were depleted with phycoerythrin (PE)–conjugated anti–Ter-119 and PE-conjugated anti-CD19 (BD) followed by separation with anti–PE-conjugated magnetic-activated cell sorter beads (Miltenyi Biotec). Cells were stained with fluorescein isothiocyanate–conjugated anti-TCRβ and PerCP–Cy5.5 anti-NK1.1 (BD), and TCRβ+ NK1.1+ cells were sorted using a FACSAria cell sorter (BD). The purity of isolated NK1.1+ T cells (MAIT cells) was >95%, as assessed by flow cytometry.

In adoptive transfer experiments, 5×10^5 MAIT cells or NK1.1– T cells (T cells) were injected intravenously into naive MR1$^{-/-}$ C57BL/6 recipient mice 1 day before administration of CII mAb. LMNCs or sorted MAIT cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine, 50 units/ml of penicillin/streptomycin, and 55 μ*M* β-mercaptoethanol (Life Technologies) and stimulated with immobilized anti-CD3 mAb (2C11, 1 μg/ml) and/or the following cytokines: IL-1β, tumor necrosis factor α (TNFα), IL-6, and transforming growth factor β (TGFβ) (all from PeproTech) and IL-23 (R&D Systems).

Detection of cytokines. Cytokine levels in the culture supernatant were determined using a sandwich ELISA. The ELISA antibodies for IFNγ were purchased from BD. Levels of IL-17 were determined using an IL-17 ELISA kit (R&D Systems).

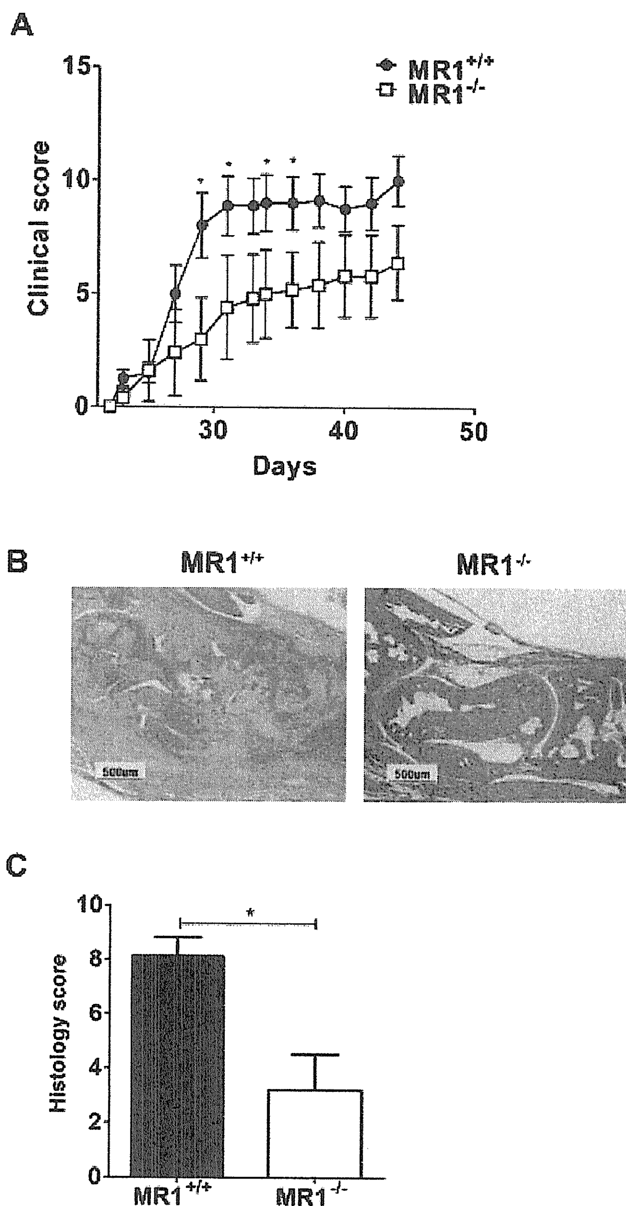

Figure 1. Amelioration of collagen-induced arthritis (CIA) in MR1$^{-/-}$ mice. **A,** Clinical scores for CIA in MR1$^{-/-}$ DBA/1J mice and in MR1$^{+/+}$ DBA/1J mice. Values are the mean ± SEM of 5–8 mice per group. * = $P < 0.05$ versus MR1$^{-/-}$ DBA/1J mice. **B,** Representative histologic sections of the joints of MR1$^{+/+}$ DBA/1J mice and MR1$^{-/-}$ DBA/1J mice. Hematoxylin and eosin stained; original magnification × 40. **C,** Histology scores in MR1$^{-/-}$ DBA/1J mice and in MR1$^{+/+}$ DBA/1J mice, expressed as the sum of the scores in the 4 paws. Results from a single representative experiment of 2 similar experiments performed are shown. Values are the mean ± SEM. * = $P < 0.05$.

Statistical analysis. Clinical or pathologic scores for CIA and CAIA in the various groups of mice are presented as

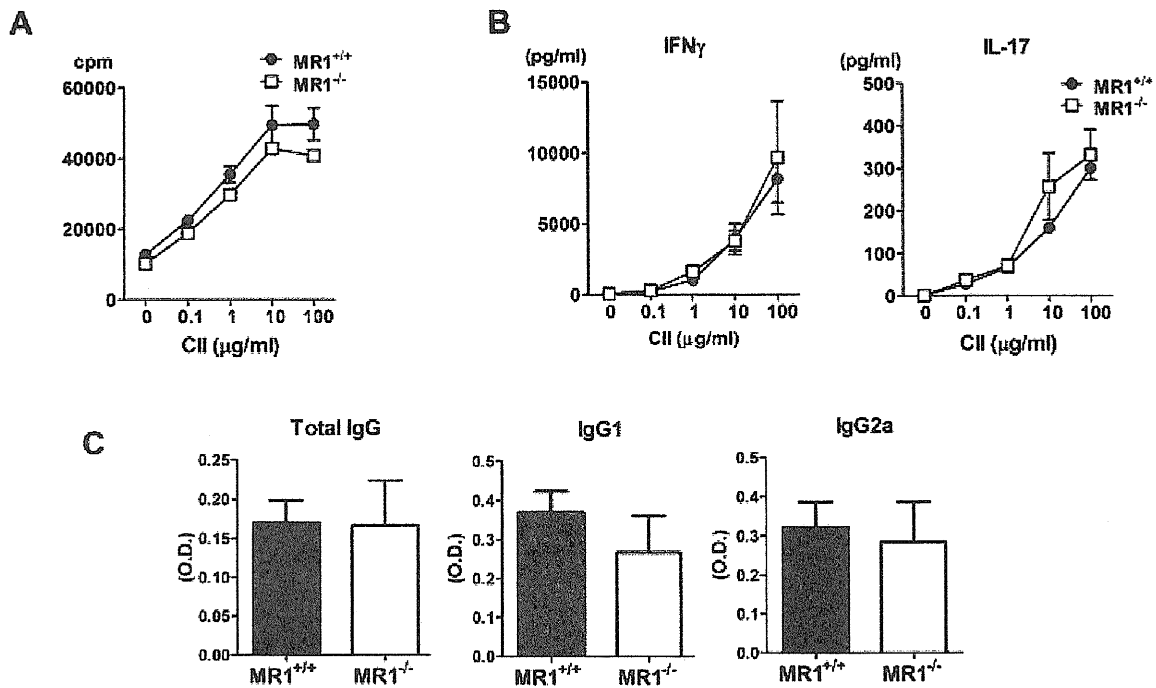

Figure 2. Type II collagen (CII) responses in MR1$^{-/-}$ DBA/1J mice. **A** and **B**, Inguinal lymph node cells from MR1$^{-/-}$ DBA/1J mice and MR1$^{+/+}$ DBA/1J mice with collagen-induced arthritis were incubated for 48 hours in the presence of CII. Proliferative responses were determined by the uptake of ^{3}H-thymidine (**A**), and the levels of interferon-γ (IFNγ) and interleukin-17 (IL-17) in culture supernatants were measured by enzyme-linked immunosorbent assay (**B**). **C**, CII-specific antibody levels in individual serum samples obtained at the end of the experiment were analyzed as described in Materials and Methods. Results from a single representative experiment of 2 similar experiments performed are shown. Values are the mean ± SEM of 5–8 mice per group. OD = optical density

the mean ± SEM clinical score for the group, and statistical differences were analyzed with a nonparametric Mann-Whitney U test. Data for cytokines and proliferation were analyzed with an unpaired *t*-test.

RESULTS

Amelioration of CIA in MR1$^{-/-}$ mice. To investigate whether MAIT cells play a role in the pathogenesis of arthritis, we first evaluated the involvement of MAIT cells in CIA using MR1$^{-/-}$ mice lacking MAIT cells. Because DBA/1J mice bearing the H-2q haplotype are the most susceptible strain for CIA, MR1$^{-/-}$ C57BL/6J mice were backcrossed to DBA/1J mice for 10 generations to obtain MR1$^{-/-}$ DBA/1J mice. Both MR1$^{-/-}$ DBA/1J mice and littermate MR1$^{+/+}$ DBA/1J mice were immunized with CII to induce CIA, and the clinical severity of arthritis was evaluated by visual scoring of each paw. As shown in Figure 1A, the clinical scores in MR1$^{-/-}$ DBA/1J mice were reduced in comparison to those in MR1$^{+/+}$ DBA/1J mice. Histologic examination of the joints of the 4 paws 44 days after CIA induction showed less cell infiltration, cartilage erosion, and bone destruction in MR1$^{-/-}$ DBA/1J mice than in the MR1$^{+/+}$ DBA/1J mice (Figure 1B). Quantification of the histologic severity of arthritis revealed that MR1$^{-/-}$ DBA/1J mice developed milder joint inflammation than MR1$^{+/+}$ DBA/1J mice (Figure 1C). These results suggest that MAIT cells contribute to the exacerbation of the disease course of CIA.

CII responses in MR1$^{-/-}$ DBA/1J mice. As the presence of MAIT cells augmented the severity of CIA, we next asked whether MAIT cells influence the CII-specific responses of T and B cells. Lymph node cells from CIA-induced animals were rechallenged with CII ex vivo. As shown in Figure 2A, the proliferative responses of lymph node cells upon stimulation with CII were similar in the two groups. Lymph node cells from both MR1$^{-/-}$ DBA/1J mice and MR1$^{+/+}$ DBA/1J mice produced comparable amounts of IL-17 and IFNγ in response to CII in a dose-dependent manner (Figure 2B). We also evaluated CII-specific immunoglobulin levels in serum obtained 35-42 days after arthritis induction. We observed a trend of reduced levels of CII-specific IgG1 in MR1$^{-/-}$ DBA/1J mice compared to the

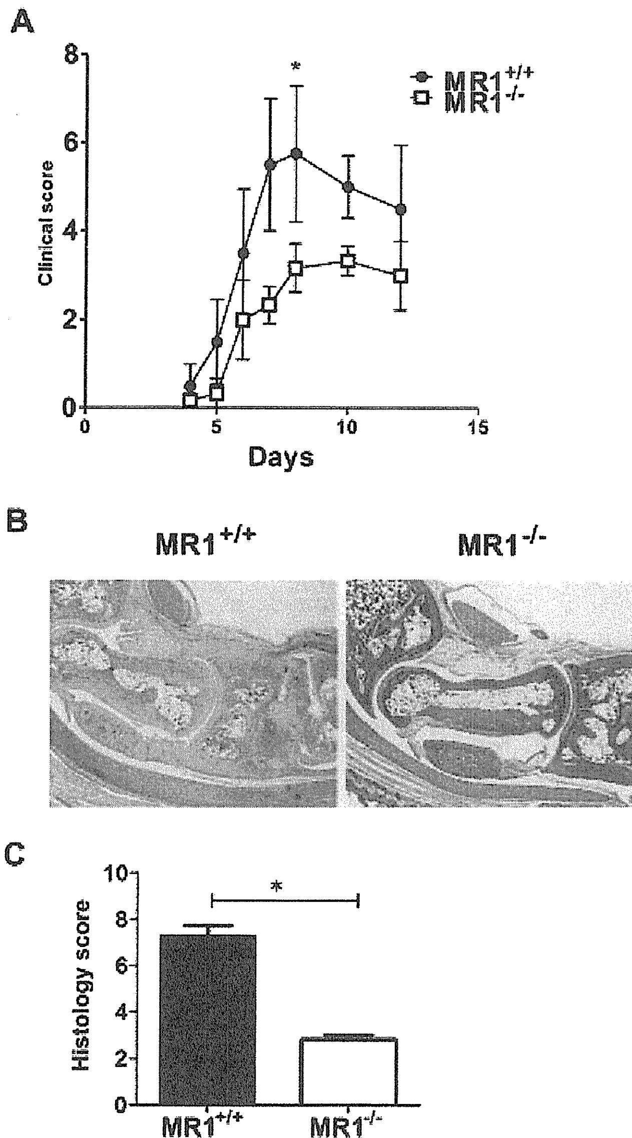

Figure 3. Amelioration of collagen antibody–induced arthritis (CAIA) in MR1$^{-/-}$ mice. **A,** Clinical scores for CAIA in MR1$^{-/-}$ C57BL/6J mice and MR1$^{+/+}$ C57BL/6J mice. Values are the mean ± SEM of 4–6 mice per group. * = $P < 0.05$ versus MR1$^{-/-}$ C57BL/6J mice. **B,** Representative histologic sections of the joints of MR1$^{+/+}$ C57BL/6J mice and MR1$^{-/-}$ C57BL/6J mice. Hematoxylin and eosin stained; original magnification × 40. **C,** Histology scores in MR1$^{-/-}$ C57BL/6J mice and in MR1$^{+/+}$ C57BL/6J mice, expressed as the sum of the scores in the 4 paws. Results from a single representative experiment of 2 similar experiments performed are shown. Values are the mean ± SEM. * = $P < 0.05$.

levels in MR1$^{+/+}$ DBA/1J mice, but the difference did not reach statistical significance (Figure 2C). These results indicate that the presence of MAIT cells has little effect on CII-specific responses.

Amelioration of CAIA in MR1$^{-/-}$ mice. The CIA model requires both adaptive and innate immune responses for disease development, and T cells and B cells responding to CII are the major players in the initiation of the disease. Although we observed significant differences in both the clinical and pathologic severity of arthritis when comparing MR1$^{-/-}$ DBA/1J mice to MR1$^{+/+}$ DBA/1J mice (Figure 1), the CII-specific responses of T and B cells appeared not to depend on the presence of MAIT cells (Figure 2). Thus, we hypothesized that MAIT cells may influence the effector phase of arthritis. To test this hypothesis, we induced CAIA in MR1$^{-/-}$ and MR1$^{+/+}$ C57BL/6J mice. By 7 days after injection of anti-CII mAb, MR1$^{+/+}$ C57BL/6J mice had developed severe arthritis, as assessed by clinical scores (Figure 3A). In contrast, the clinical scores in the MR1$^{-/-}$ C57BL/6J mice were lower compared to those in the MR1$^{+/+}$ C57BL/6J mice. Histologic assessment 10 days after arthritis induction revealed severe arthritis with leukocyte infiltration, synovial hyperplasia, pannus formation, cartilage erosion, and bone destruction in MR1$^{+/+}$ C57BL/6J mice, whereas these features were milder in MR1$^{-/-}$ C57BL/6J mice (Figures 3B and C).

Augmentation of arthritis in MR1$^{-/-}$ mice by adoptive transfer of MAIT cells. To demonstrate that MAIT cells actually enhance disease severity in the

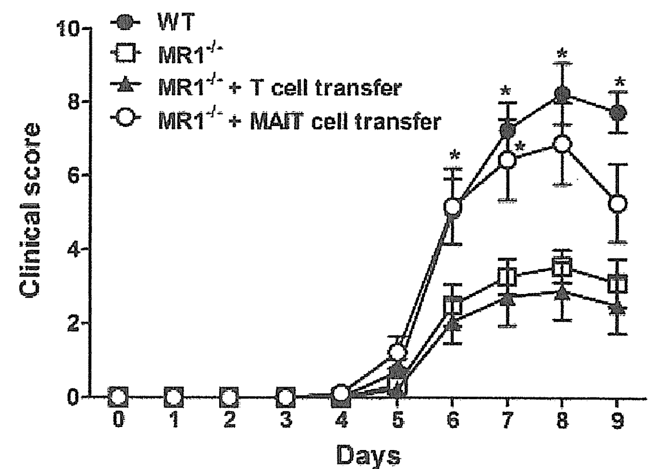

Figure 4. Augmentation of arthritis by adoptive transfer of mucosal-associated invariant T (MAIT) cells in MR1$^{-/-}$ mice. MR1$^{-/-}$ C57BL/6J mice received 5 × 10^5 NK1.1+TCRβ+ T cells (MAIT cells) or an equal number of NK1.1–TCRβ+ cells (T cells) from V$_{\alpha}$19-transgenic CD1d1$^{-/-}$ mice. One day later, collagen antibody–induced arthritis was induced in wild-type (WT) C57BL/6J mice, MR1$^{-/-}$ C57BL/6J mice, and MR1$^{-/-}$ C57BL/6J mice reconstituted with T cells or MAIT cells. Results pooled from 2 similar experiments performed are shown. Values are the mean ± SEM of 8–10 mice per group. * = $P < 0.05$ versus MR1$^{-/-}$ C57BL/6J mice.

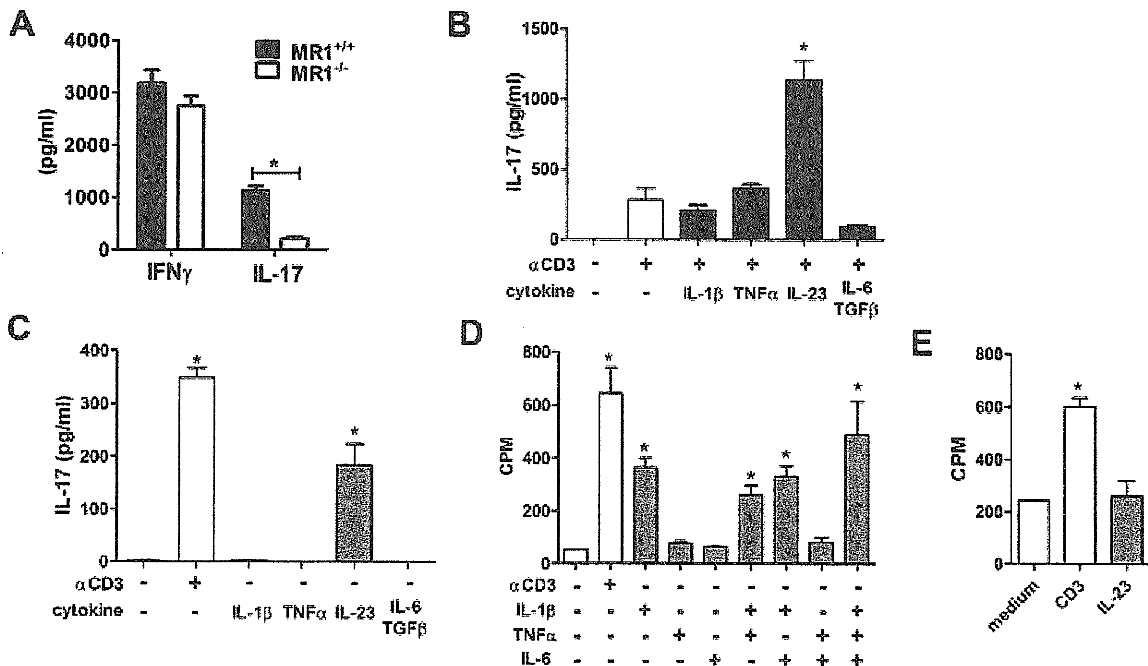

Figure 5. Cytokine-mediated mucosal-associated invariant T (MAIT) cell activation. **A,** Liver mononuclear cells from $MR1^{+/+}$ C57BL/6J mice and $MR1^{-/-}$ C57BL/6J mice were stimulated for 48 hours with immobilized anti-CD3 (αCD3) monoclonal antibody (mAb). The levels of interferon-γ (IFNγ) and interleukin-17 (IL-17) in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). * = $P < 0.05$. **B,** MAIT cells were stimulated for 48 hours with immobilized anti-CD3 mAb, with or without IL-1β, tumor necrosis factor α (TNFα), IL-23, or IL-6 plus transforming growth factor β (TGFβ), and the levels of IL-17 were measured by ELISA. * = $P < 0.05$ versus anti-CD3 mAb stimulation alone. **C,** MAIT cells were stimulated with immobilized anti-CD3 mAb or the indicated cytokines, and IL-17 levels were measured. * = $P < 0.05$ versus unstimulated control. **D** and **E,** Proliferative responses after 48 hours of exposure to the indicated cytokines were determined as the uptake of ^{3}H-thymidine. Results from a single representative experiment of 2 similar experiments performed are shown. * = $P < 0.05$ versus unstimulated control. Values in **A–E** are the mean ± SEM.

CAIA model, we performed adoptive transfer experiments. Most NK1.1+ TCRβ T cells within liver lymphocytes from $CD1d1^{+/+}$ mice are iNKT cells, and we and other investigators previously demonstrated that the NK1.1+ TCRβ T cell population in $V_{\alpha}19i$-transgenic $CD1d1^{-/-}$ mice is highly enriched in $V_{\alpha}19i$ TCR+ cells (15,17). Thus, to obtain MAIT cells, we isolated NK1.1+ TCRβ T cells from $V_{\alpha}19i$-transgenic $CD1d1^{-/-}$ mice. We adoptively transferred these MAIT cells into $MR1^{-/-}$ C57BL/6J mice, and 1 day later, we injected these mice with anti-CII mAb to induce CAIA. $MR1^{-/-}$ C57BL/6J mice reconstituted with MAIT cells developed severe arthritis at a level similar to that of wild-type (WT) C57BL/6J mice (Figure 4). However, the transfer of an equal number of T cells into $MR1^{-/-}$ C57BL/6J mice had little effect on the clinical arthritis scores. Taken together, these results suggest that the presence of MAIT cells augmented arthritis mainly by enhancing the inflammation in arthritis.

Cytokine-mediated MAIT cell activation. To understand the mechanism by which MAIT cells exacerbate the disease course of arthritis, we first compared the cytokine-producing capacity of T cells from $MR1^{-/-}$ and WT C57BL/6J mice. Upon anti-CD3 mAb stimulation, LMNCs from $MR1^{-/-}$ and WT C57BL/6J mice produced comparable amounts of IFNγ. However, the level of IL-17 was lower in $MR1^{-/-}$ C57BL/6J mice than in WT C57BL/6J mice (Figure 5A).

It was recently demonstrated that human MAIT cells express the Th17-associated transcription factor retinoic acid receptor–related orphan nuclear receptor (ROR) and produce high levels of IL-17 (33). We therefore sought to determine whether mouse MAIT cells produce IL-17, which is known to play a pathogenic role in arthritis. MAIT cells were sorted from LMNCs obtained from $V_{\alpha}19i$-transgenic $CD1d1^{-/-}$ mice and were stimulated ex vivo with anti-CD3 mAb. As previously shown (34), MAIT cells produced large amounts of IL-17. In addition, IL-17 production by anti-CD3 mAb–stimulated MAIT cells was augmented in the presence of IL-23 (Figure 5B).

Innate-like lymphocytes such as iNKT cells and

γ/δ T cells are known to be activated by cytokines directly, without TCR stimulation. A combination of IL-12 and IL-18 activates iNKT cells to produce IFNγ, and IL-1 together with IL-23 induces IL-17 production by γ/δ T cells (31,35,36). We therefore next asked whether MAIT cells are activated directly by cytokines. MAIT cells were incubated with various cytokines without TCR stimulation, and cytokine concentrations in the culture supernatants were evaluated. Surprisingly, MAIT cells produced high levels of IL-17 after exposure to IL-23 in the absence of TCR stimulation (Figure 5C).

Inflammatory cytokines such as IL-1β, TNFα, and IL-6 play critical roles in arthritis models and in human RA. Therefore, we next tested whether MAIT cells could be activated by these cytokines. As shown in Figure 5D, IL-1β induced robust proliferation of MAIT cells, although cytokine production was not observed after exposure to these cytokines, including IL-1β (data not shown). In addition, IL-23 did not induce proliferation of MAIT cells (Figure 5E). Thus, in the absence of TCR stimuli, IL-1β induced the proliferation of MAIT cells and IL-23 promoted the production of IL-17 by MAIT cells.

DISCUSSION

Previous studies by our group as well as others revealed that iNKT cells play pathogenic roles in CIA and CAIA by inducing a Th1 or Th17 shift of autoimmune T cells and by augmenting the inflammation in arthritis (25–27). In the present study, we demonstrated that MAIT cells contribute to the severity of CIA and CAIA mostly by augmenting joint inflammation during the effector phase of arthritis. $MR1^{-/-}$ mice were originally generated on the 129P2 background. Although $MR1^{-/-}$ mice were backcrossed onto C57BL/6 or DBA/1J, we are not able to exclude the possibility that some residual sequence from the 129P2 mice affects the arthritis susceptibility of $MR1^{-/-}$ C57BL/6 and $MR1^{-/-}$ DBA/1J mice. However, since the reconstitution of MAIT cells induced severe CAIA in $MR1^{-/-}$ C57BL/6 mice, the phenotype observed in $MR1^{-/-}$ mice seems to be dependent on the lack of MAIT cells.

It has been revealed that there are CD1d-restricted T cells that are different from iNKT cells and do not express an invariant TCRα chain ($V_\alpha14$–$J_\alpha18$ in mice and $V_\alpha24$–$J_\alpha18$ in humans). Such CD1d-restricted T cells are called type II NKT cells and possess different functions from iNKT cells. Recently, CD1d-restricted NKT cells, which recognize murine type II collagen peptide 707–721, were reported to suppress CIA (37). It is not known whether there are distinct subsets with different functions among MAIT cells or whether there are other T cells that are restricted by the MR1 molecule. As adoptively transferred $V_\alpha19i$ T cells augmented CAIA in $MR1^{-/-}$ mice, MAIT cells include the population that enhances the inflammation in arthritis.

It was recently shown that IL-17–producing γ/δ T cells were observed in the joints of mice with CIA and that blocking a certain subset of IL-17–producing γ/δ T cells suppressed CIA (29). However, γ/δ T cells have been shown to be dispensable for the development of CIA (38). In addition, anti-CII–specific antibody levels were comparable between γ/δ T cell–deficient and wild-type mice. These findings suggest that MAIT cells and γ/δ T cells share similar roles in arthritis and that both are involved mainly in the effector phase of arthritis. It is known that γ/δ T cells as well as iNKT cells are increased during CIA. Because MAIT cells share similar features with γ/δ T cells and iNKT cells, MAIT cells may also be increased during CIA.

We observed a significant decrease in IL-17 production by LMNCs upon stimulation with anti-CD3 mAb in $MR1^{-/-}$ mice compared to WT control mice. As sorted MAIT cells produced high amounts of IL-17 after anti-CD3 mAb stimulation, the major source of IL-17 responsible for the difference between $MR1^{-/-}$ and WT mice seems to be MAIT cells. Th17 cells and iNKT cells have been shown to produce IL-21, which enhanced IL-17 production or induced proliferation of IL-17–producing cells (39). It is not known whether MAIT cells produce IL-21, but MAIT cells might augment IL-17 production by other LMNCs, including γ/δ T cells, through such mechanisms. Further studies to determine whether MAIT cells regulate γ/δ T cells under both physiologic and pathologic conditions, including in the presence of arthritis, will be of interest.

The frequency of murine γ/δ T cells is 1–5% in blood lymphocytes and 25–60% in gut lymphocytes. Human γ/δ T cells also comprise up to 2–3% of peripheral T cells (9,10). Although the precise frequency of murine MAIT cells is not known, it has been speculated that MAIT cells may comprise up to 10% of double-negative T cells in the gut lamina propria and <2% of double-negative T cells in the mesenteric lymph nodes, indicating that the frequency of murine MAIT cells is much lower than that of mouse γ/δ T cells (15). It has been suggested that γ/δ T cells are the predominant source of IL-17 in the joints of CIA mice, but IL-17–producing γ/δ T cells could not be detected in RA synovial tissue (31). Recently, Martin et al (23) revealed that human MAIT cells can be identified as $V_\alpha7.2+$ $CD161^{high}$ T cells, which are abundant in blood. In addition, human MAIT cells produce IL-17 and express

tissue-homing chemokine receptors (23). An IL-17–producing CD161^{high} T cell population has been described in human arthritic joints (40). Thus, it is possible that MAIT cells rather than γ/δ T cells play a major role in the pathogenesis of human RA.

CD4+ Th17 cells require IL-6/STAT-3 activation for the expression of RORγt, which is a crucial transcription factor for IL-17 production (41). However, some innate-like lymphocyte subsets, such as iNKT cells, γ/δ T cells, and lymphoid tissue–inducer (LTi)–like cells, are known to constitutively express RORγt, IL-1 receptor type I, and IL-23R (42). In addition, these IL-17–producing innate-like lymphocytes, including LTi cells, γ/δ T cells, and iNKT cells, secrete IL-17 when stimulated by IL-23 with or without IL-1β. In this study, we demonstrated cytokine-mediated activation of MAIT cells. MAIT cells produced IL-17 in response to IL-23. Moreover, IL-1β induced proliferation of MAIT cells. Thus, it is possible that MAIT cells may contribute to the disease progression of arthritis through another mechanism in addition to IL-17 production. In adoptive transfer experiments, MAIT cells augmented the disease severity of CAIA in MR1-deficient mice. Thus, this result also indicates that MAIT cell–mediated exacerbation of arthritis may be induced by cytokines, without a requirement for TCR stimulation.

In EAE, disease suppression by MAIT cells was accompanied by a reduction in the production of cytokines, including IFNγ and IL-17, by T cells and increased IL-10 production by B cells. Encephalitogenic T cells play a major role in EAE (43,44). EAE can be induced in naive mice by transferring myelin-reactive T cells. T cell–targeted therapies, including anti–very late activation antigen 4 treatment, have been shown to suppress EAE. Although CIA was reduced in MR1$^{-/-}$ DBA/1J mice, we observed a significant decrease in CII-specific IgG1 antibody levels in these mice as compared with their WT controls in some experiments (data not shown), suggesting the inhibition of Th1 responses by MAIT cells. Therefore, it is still possible that MAIT cells suppress Th1 response during the early induction phase of CIA. MAIT cells may be functionally plastic, and thus exert different functions depending on the pathologic condition. Arthritis involves massive cytokine production due to various types of immune cell activation. Since MAIT cells can be activated by inflammatory cytokines, MAIT cells may contribute to augment the immune response once overt inflammation occurs.

In summary, we have shown that MAIT cells contribute to the progression of arthritis by enhancing the inflammation in CIA and CAIA models. In addition, we demonstrated that MAIT cells could be activated by cytokine stimulation even without TCR stimulation. We and others previously reported that, although iNKT cells play pathogenic roles in arthritis models, modulation of iNKT cell function by ligands successfully suppressed arthritis (45–47). The proportion of human MAIT cells appears to be much higher than that of mouse MAIT cells. Therefore, MAIT cells may play an important pathogenic role in human arthritis and MAIT cell–targeted therapy may hold promise as a new therapeutic intervention for arthritis, including RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Miyake had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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