Antigen-independent IL-17A production by bystander-activated CD4+IL-1R1+ cells in patients with Multiple Sclerosis

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Directed by Professor Eun Jig Lee

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ABSTRACT

Antigen-independent IL-17A production by bystander-activated CD4+IL-1R1+ cells in patients with Multiple Sclerosis

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(Directed by Professor Eun Jig Lee)

Multiple sclerosis (MS) is a demyelinating disease caused by an autoantigen recognizing CD4+ T cells. However, IL-17A-producing CD4+ T cells that are bystander-activated by IL-1β and IL-23, and T cell receptors (TCR) independently, could contribute to experimental autoimmune encephalomyelitis. Here, we studied the differences in the frequency and function of bystander-activated CD4+ T cells in patients with MS. A significantly higher frequency of CD4+IL-1Rl+ T cells was found in memory cells than in naïve CD4+ T cells and in Th17/Th17.1 than in Th1/Th2 subtypes in both MS and healthy controls (HC). Following IL-1β and IL-23 stimulation, IL-1Rl expression was markedly increased in both memory and Th17/Th17.1 cells, and their IL-17A-production was increased after bystander activation, which was significantly higher in MS compared with HC. Our study suggests a potential role of IL-17A-producing bystander-activated CD4+IL-1Rl+ T cells in MS.

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I.   INTRODUCTION

1.   Multiple Sclerosis

A.   Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, immune-mediated disease of the central nervous system (CNS), where activated, autoreactive T cells migrate into the CNS, mediating inflammation [1]. The biggest feature of MS is inflammatory demyelination that occurs in the CNS. Inflammation occurs in all phases of MS, and it is more severe in the acute stage than in the chronic stage [2]. This phenomenon causes a range of MS symptoms, different for each patient, such as vision problems, diplopia, dizziness, depression, slurred speech, and fatigue [3]. The precise pathogenesis of MS is not fully uncovered, but it is known to be a complex disease driven by a variety of factors. It is presumed to be the result of complex interactions between genetic, environmental, and immunological events [2, 4, 5]. MS is now becoming a global issue, affecting more than 2.8 million people worldwide [6]. The prevalence, incidence and age-standardized disability-adjusted life-year (DALY) of MS markedly increased over the last few decades [7, 8]. The incidence rate by gender is around twice as high in females as in males [6]. Accordingly female predominance was also noted in MS prevalence [9].

B. Pathogenesis of Multiple Sclerosis

The long-favored hypothesis of MS pathogenesis is that autoreactive immune cells generated outside the CNS migrate into the CNS through damaged blood brain barrier (BBB) and induce an inflammatory cascade which leads to demyelination and neuronal damage. It is yet to be defined how these autoreactive immune cells are generated. One of the most widely accepted theory is 'molecular mimicry': antigenic determinants share the same structural and phenotypic characteristics as foreign antigens upon infection, which are produced by cross-reaction through contacted motif between the major histocompatibility complex (MHC) and T cell receptor (TCR) on lymphocytes [10]. Representative MS autoantigens include various myelin-related proteins such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) [11, 12, 13, 14, 15]. In addition, non-myelin proteins such as B crystalline, and neural proteins such as contactin-2 are also identified, which can induce auto-reactive immune responses in MS [16, 17].
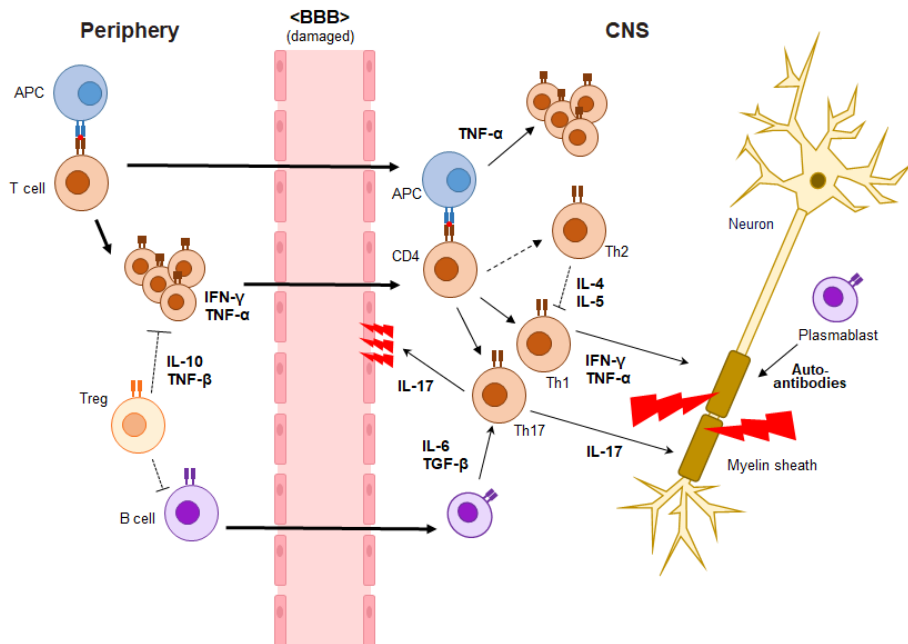
Figure 1. Pathogenesis of MS. The pathogenesis of autoreactive T cells in CNS of MS. T cells are primed with APC in the lymph node, and this activates T cells to invade the CNS throughout damaged BBB. They activate macrophages and microglia, causing them to release cytokines that promote inflammatory responses and demyelination.

C.   Diagnosis of Multiple Sclerosis

MS is diagnosed by the characteristic clinical signatures and magnetic resonance imaging (MRI) according to McDonald criteria revised in 2017, [18]. Although not specific, cerebrospinal fluid (CSF) oligoclonal bands (OCBs) are the supportive findings for the diagnosis of MS and also one of the criterions for dissemination in time. OCBs are the bands of immunoglobulins in response to antigen exposure and CSF-restricted OCBs indicate intrathecal synthesis of immunoglobulins. Up to 95% of patients with MS show the occurrence of abnormal OCB synthesis [18, 19, 20].

D.   Subtypes of Multiple Sclerosis

MS is categorized into 4 populations: relapsing-remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS), and progressive relapsing (PRMS) [21, 22]. RRMS is the most common phenotype of MS that affects almost 85% of total MS patients. It's typically defined by an acute exacerbation of neurological symptoms with partial or full recovery followed by a period of remission [23, 24]. Often, this relapsing-remitting course is followed by a phase of insidious worsening of neurologic function independent from relapses that is termed SPMS. One of the major differences between RRMS and SPMS is the response to available treatments. Until recently, no treatments with demonstrated efficacy in terms of preventing disability worsening were available for SPMS. [25, 26]. PPMS, which accounts for 10% of all MS patients, is identified by gradual neurological disability from the beginning of the symptoms without any early relapse or remission [21, 27]. PRMS, which represents only about 5% of MS patients, is defined by a steady worsening of the disease from the onset with acute relapses but no

remission. The disease condition can either improve or remain stable in this situation [28].

E. Treatment of Multiple Sclerosis

Treatment of MS includes diseases modifying therapies (DMTs), acute relapse treatment, comorbidity management, symptom control, appropriate rehabilitation, and lifestyle modifications.

DMTs for MS aim to decrease the frequency of relapses and reduce long-term disability. Since the first DMT, interferon beta-1b, was approved by the Food and Drug Administration (FDA) in 1993, more than 10 DMTs have been released and used in clinical practice [29, 30]. Those DMTs modulate the immune system through mechanisms that include suppression of immune cells, sequestration of lymphocytes in lymph node, TH1/TH2 shift, and/or interference with DNA synthesis in lymphocytes [31]. Injectable DMTs (Interferons, Glatiramer acetate) reduce the further relapse rate by approximately 30% compared with placebo [30], and are generally considered to be safer than other DMTs in terms of infection rate [32]. However, frequent self-injections, injection site reactions and flu-like symptoms are main limitation for continuing this type of DMTs [33].

Oral forms vary in their effects on relapse reduction, but these are regarded as being more effective than injectable forms [34]. Teriflunomide interrupts the mitochondrial enzyme involved in de novo pyrimidine synthesis dihydroorotate dehydrogenase [35]. Although exact mechanism of action has not fully revealed yet, Dimethyl fumarate has been associated with activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway [36]. Fingolimod, Siponimod, Ozanimod and Ponesimod are S1P modulators by binding to one of S1P receptors, resulting in internalization

of the receptor and sequestration of lymphocytes in lymph nodes [37]. Cladribine is a purine analogue affecting on rapidly proliferating cells, leading to cell death [38].

There are also intravenous infusion forms. Natalizumab, a monoclonal antibody, binds selectively on the α4 integrin subunit expressed on the surface of lymphocytes, preventing the entrance of lymphocytes into the CNS [39, 40]. Alemtuzumab is a monoclonal antibody that selectively binds CD52 of lymphocytes, resulting lymphocyte depletion [41]. CD20 monoclonal antibodies (Ocrelizumab, Ofatumumab) selectively bind B-cells that express the CD20 antigen, which leads to cell destruction by both cell-mediated and complement dependent cytotoxicity [42, 43].

As the main mechanism of all above-mentioned DMTs is thought to be suppressing neuroinflammation in CNS, DMTs are usually approved to treat RRMS or active SPMS, which have the process of active neuroinflammation in CNS [44]. In contrast, PPMS has been regarded as having less neuroinflammation. Thus, only one DMT has been approved for treatment of PPMS, Ocrelizumab, which is hypothesized to reduce B-cell–mediated inflammation that can make progressive neurodegeneration [44].

2. T helper 17 cells in Multiple Sclerosis

MS has been generally assumed to be an autoimmune disease mediated by CD4+ T helper cells (Th). Traditionally, in adaptive immunity, responses of T cells are considered successful when antigen-specific activation leads to the creation of clonal expansion and memory [45]. CD4+ T cells respond to their specific antigen and differentiate into various helper T cells, and functionally separated into numerous subtypes that are distinct in terms of their recognition of transcription factors and cytokines [46, 47]. The fundamental commitment of CD4+ T cell subsets is exceedingly precise, but not permanent. These shifting behaviors affect the aberrant release of pro-inflammatory and anti-inflammatory cytokines, which may exacerbate the progression of autoimmune diseases including MS [46, 48]. In this case, T helper 17 (Th17) cells are believed to be one of the key players in the immunopathogenic process of MS, mediating neuroinflammation [1, 49]. Th17 is divided into non-pathogenic and pathogenic types. When naïve CD4+ T cells stimulated by TGF-β and IL-6 secrete IL-17 and IL-10 and become non-pathogenic Th17 cells [50]. After additional stimulation with IL-1β and IL-23, Th17 expresses pathogenic cell signature genes such as ROR-gamma-t (ROR-γt) and T-bet and contribute to the disease exacerbation by producing IL-17, IL-21, and IL-22 [51, 52, 53]. Pathogenic Th17 cells can destroy the tight junctions of BBB by infiltrating the CNS and releasing IL-17, [54, 55] which further induces astrocytes and microglia in the CNS to produce inflammatory mediators [56, 57]. Endothelial cells respond to high levels of IL-17 by releasing the chemokines CXCL1 and CCL2. Leukocyte adhesion and migration through the BBB are controlled by CCL2, which leads to a BBB disruption in a positive feedback loop [58]. In addition, by secreting IL-17A and inducing the production of chemokines that attract neutrophils from various cell types in the central nervous system trigger

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an immune response [59]. When Th17 cells were depleted from EAE mice, the remaining T cells no longer exacerbated EAE after adoptive transfer, and the severity of EAE was effectively reduced after IL-17 monoclonal antibody treatment [60]. The infiltration of mononuclear cells into spinal cord is reduced in EAE of IL-17$^{-/-}$ mice [61]. These results showed that IL-17 is important in the activation of encephalitogenic T cells in EAE.

In human studies, it was demonstrated that the frequency of Th17 cells was elevated in the peripheral blood of MS patients [55, 62, 63]. IL-17 mRNA, IL-17+ T cell levels, and IL-17 gene expressions were also higher in MS brain lesions [64, 65] . In the same context, IL-17 was increased in serum and CSF in MS patients [66, 67, 68].
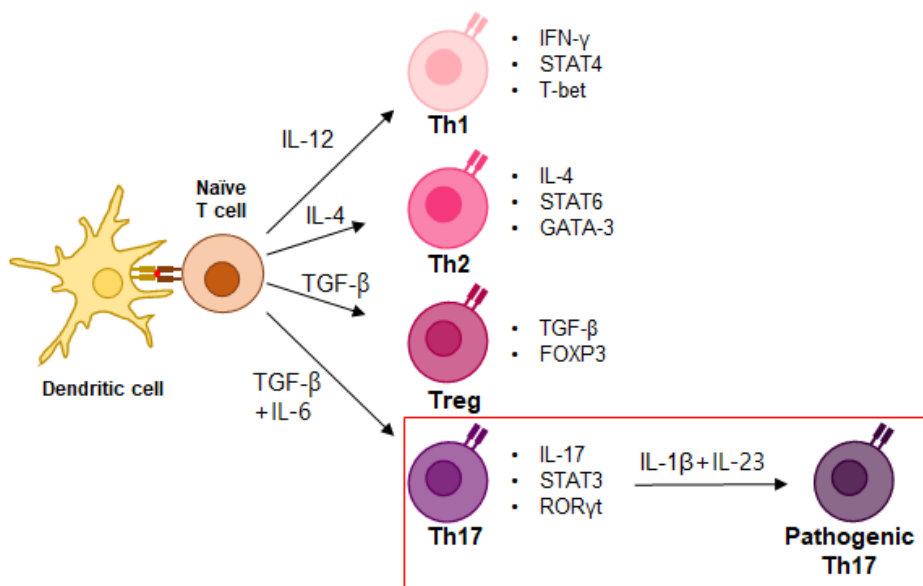
Figure 2. Helper T cell differentiation. Naive T cells are divided into several subtypes according to the releasing and recognizing cytokines after TCR stimulation. Among them, the cell type that is stimulated by TGF-β and IL-6 and secretes IL-17 is called Th17 cell. When IL-1β and IL-23 stimulation are additionally added, RORγt and T-bet gene are expressed and become pathogenic Th17 cell.

3. Bystander-activated CD4+ T cells in Multiple Sclerosis

A. Innate Lymphoid Cells

Infiltration and activation of innate and adaptive immune cells are the major characteristics of tissue inflammation. Innate immunity is the "first line of defense" against the host, a system in which innate immune cells produce cytokines and chemokines or respond to risk signals to lead APC activation [69]. The adaptive immunity is comprised of cell-mediated and humoral immunity, which are antigen-specific mechanisms of activating T and B cells that involve antigen recognition, immune checkpoint, cytokine stimulation, and metabolite-associated signal recognition [69]. Thus, lymphocytes have been accepted as mediators of adaptive immunity upon antigen recognition. However, recent discoveries found the innate-like function of lymphocytes and lymphocytes with such function are termed innate lymphoid cells (ILC) [70, 71, 72]. Unlike antigen-specific CD4+ T cells, ILCs are resident in nonlymphoid tissues [73], lack antigen receptors, do not require co-stimulation, and are instead activated by soluble mediators such as proinflammatory cytokines, lipids, hormones, etc. [74]. The minimal activation requirements of ILCs make them excellent first responders during the early stage of the immune response, as well as the immediate source of effector cytokines in response to the disruption of homeostasis. ILCs express and produce the same transcription factors and cytokines as effector T cells (Th1, Th2 and Th17) and are derived from common lymphoid progenitors. This finding implies that differentiated T cells may have an innate-like capacity and these "bystander-activated" antigen non-specific T cells may have a role in autoimmune diseases [75].

B. Bystander-activated CD4+ T cells in Multiple Sclerosis

Recent studies have highlighted the importance of bystander-activated

cells without antigen recognition along with antigen-specific T cells in the pathogenesis of various diseases including autoimmune disease, cancer, or infection[75]. In the EAE, CNS T-cells were retained regardless of antigen specificity once T cell retention within the CNS was initiated by myelin-specific T cells [76]. Study on MOG-induced EAE showed that the majority of infiltrating effector CD4+ T cells in the spinal cord were not specific to MOG [76, 77, 78]. This means that antigen non-specific CD4+ T cells contribute to the pathogenesis of autoimmune diseases in response to the cytokines, without TCR reaction. Accordingly, it was found that some differentiated CD4+ T cells generate inflammatory cytokines by STAT activators (IL-23, IL-12, IL-2) and IL-1 family members (IL-1β, IL-18, IL-33) [79]. These MOG non-specific T cells produce high levels of effector cytokines such as IL-17A and were able to induce EAE when bystander-activated with IL-1β and IL-23 in the absence of TCR signaling [72, 80]. In addition, bystander-activated CD4+ T cells can also produce IFN-γ and GM-CSF [77]. These findings indicated that CD4+ T cells can be bystander-activated in response to pro-inflammatory cytokines and produce effector cytokines like innate-like lymphocytes. These bystander-activated CD4+ T cells are memory type (CD4+CD25-CD45RA-) and express IL-1β receptor 1 (IL-1R1) in EAE [77]. It is also known from $il1r1^{-/-}$ mice experiments that IL-1R1 can induces Th17 cells to have pathogenic functions that produce IL-17 even without TCR reaction. [81]. More importantly, the pathogenic function of bystander-activated CD4+ T cells that contributes to EAE pathogenesis was found to be IL-1R1-dependent [77, 81]. Likewise, in healthy humans, bystander-activated (memory-typed IL-1R1+) CD4 T cells produce inflammatory cytokines after IL-7, IL-1β, and IL-23 stimulation in TCR independent manner [77].

These results indicate that CD4+ T cells with significant effector functions of producing IL-17A via bystander activation have the potential for

autoimmune neuroinflammation. However, until now, these bystander-activated CD4+ T cells have only been studied in the EAE model, not in MS patients. Although the EAE model is well known as a representative animal model of autoimmune disease, it is necessary to verify it in patients because not all immune systems are the same. Moreover, human studies exist only on the evaluation of the expression of IL-1R1 on bystander-activated CD4+ T cells in a limited number of healthy individuals. Hence, we aimed to investigate the differences in the frequency and function of these bystander-activated CD4+IL-1R1+ T cells in MS.

II. MATERIALS AND METHODS

1. Study subjects

This study included 28 patients with MS from the National Cancer Center (13 men and 15 women, mean of age 33 ± 9 years). Among these patients, 20 were treated with interferon-β (IFN-β) and 8 were treated with natalizumab (NTZ). We chose the samples from the patients with IFN-β or NTZ treated MS, an immunomodulatory treatment, to set aside errors caused by direct inhibition of cells due to immunosuppressive treatment. The diagnosis of MS was based on the 2017 McDonald criteria [18]. 21 age- and sex-matched healthy volunteers (11 men and 10 women, mean age of 30.3 ± 6.8 years) were included as controls. Demographic and clinical information of the study population is summarized in Table 1. The study was approved by the Institutional Review Board of National Cancer Center (NCC2020-0343). Written informed consent was obtained from all participants.

Table 1. Clinical characteristics of the study population

Characteristic		MS (n=28)	HC (n=21)
Age		33.2 ± 9	30.3 ± 6.8
Sex (n)	Male	13	10
	Female	15	11
Onset age, mean ± SD (years)		26.5 ± 7.26	-
Disease duration, mean ± SD (years)		7.96 ± 5.76	-
Expanded disability status scale (EDSS), mean ± SD (years)		2.17 ± 1.86	-
Treatment (n)	IFN-β	20	-
	NTZ	8	
Treatment duration, mean ± SD (years)		6.75 ± 4.99	-

Disease durations were calculated as years passed from the onset year to the sampling year. The treatment duration was defined as the years between the start date of medications and the sampling date.

2. Peripheral blood mononuclear cells (PBMC) isolation

PBMC were isolated from patients with MS, and healthy controls (HC) using a Ficoll-Paque Plus (GE Healthcare, Little Chalfont, UK) gradient centrifugation. The blood was mixed with 2mM ethylenediaminetetraacetic acid (EDTA)-1% phosphate-buffered saline (PBS) at a 1:1 ratio in a 50ml conical tube (Falcon). After adding 15ml of Ficoll-Paque Plus solution in a separate 50ml tube, the blood mixture slowly added on the top of Ficoll-Paque Plus solution to separate the layers. The tube was centrifuged at 1640 rotation-per-minute (rpm) for 30 minutes(mins) with the brakes off. The buffy coat layer was collected and mixed with 2mM EDTA-PBS and centrifuged at 1600rpm for 20 mins. The cell pellet was resuspended with 2mM EDTA-PBS and centrifuged again for the second wash. Isolated PBMC was either directly assayed or cryopreserved.

3. MACS separation

CD4+ T cells were subsequently separated by magnetic column purification using anti-CD4-coated beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The freshly isolated PBMC was centrifuged at 300xg for 10 mins and the supernatants was removed. The cell pellet was resuspended in 80μl of MACS buffer (0.5 bovine serum albumin (BSA) + 1X PBS) per 1×10^7 cells. 20ul of CD4 MicroBeads per 10^7 cells were added and incubated at $2-8°C$ for 15 mins. The cells were resuspended and wash with MACS buffer

and centrifuged at 300xg for 10 mins. The supernatant was aspirated, and the pellet was resuspended in 500ul of MACS buffer. Cell mixture was applied to the MS column that was placed in the magnetic field of MiniMACS Sperator (Miltenyi Biotec). The unlabeled, CD4- cells that passed through were collected in a 15ml tube. Labeled CD4+ cells were also collected in another 15ml tube by removing a column from the magnetic field and flushing out the cells with the MACS buffer by firmly pushing the plunger into the column.
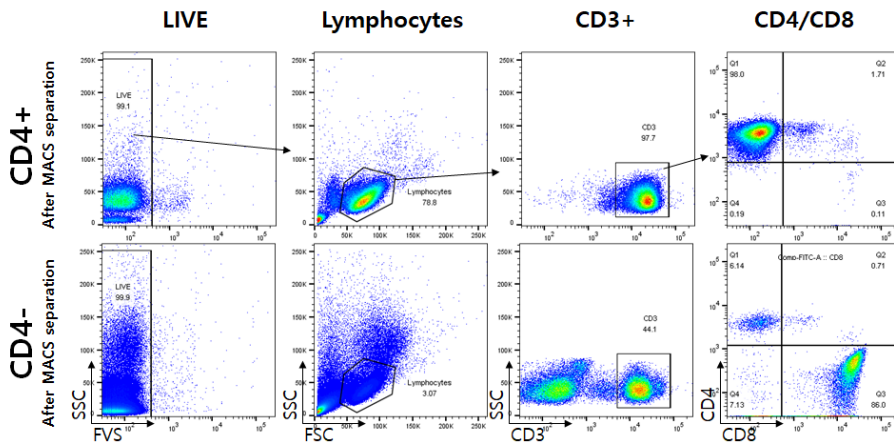
Figure 3. Quality check after CD4+ MACS separation. Fresh PBMC was separated by magnetic column purification, using anti-CD4-coated beads. After MACS sorting, the labeled cells (CD4+) and unlabeled cells (CD4-) were stained with fluorochrome-conjugated mAbs against Alexa Fluor700-FVS, BUV395-CD3, BUV496-CD4, and FITC-CD8 to verify the cells were separated.

4. Bystander activation

Purified cells were cultured in complete RPMI 1640, L-glutamine, and NaHCO3, supplemented with 10 % fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and penicillin/streptomycin (100 U/mL) (Hyclone). For *in vitro* culture of bystander-activation of CD4+ T cells, 2×10^5 sorted CD4+ T cells were stimulated with IL-1β (20ng/mL, R&D Systems, Minneapolis, MN, USA) and IL-23(20ng/mL, R&D Systems) for 3days into round-bottom 96-well plate. IL-7 (10ng/mL, R&D Systems) was also added for T cell maintenance (RM et al., 2003).

5. Intracellular cytokines staining (ICS)

The production of cytokines was detected by ICS. Phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma-Aldrich, Burlington, MA, USA), ionomycin (500 ng/mL, Sigma-Aldrich), and Golgistop (BD Biosciences, San Diego, CA, USA) were added 6 hours before cell culture terminated. The cells were harvested and stained with Fixable Viability Stain-Alexa Fluour 700 according to the manufacturer's instructions (BD Biosciences) to determine live and dead cells. For the immunophenotyping, cell surfaces were stained with fluorochrome-conjugated monoclonal antibodies (mAbs) below (Table 2). Identical surface staining steps were performed with freshly isolated PBMC before culture (ex vivo surface staining). Memory and naïve CD4+ T cells were defined by their selective expression of CD45RA, and Th1, Th2, Th17, and Th17.1 cells were

categorized according to the expression of CXCR3 and CCR6 in CD4+CD25- cells. Intracellular cytokine staining (ICS) was performed to detect cytokine production. Cells were washed after fixation and permeabilization (BD Biosciences), and subsequently stained with fluorochrome-conjugated antibodies against IL-17A-BV650, interferon (IFN)-γ-BV605, and granulocyte-macrophage colony-stimulating factor (GM-CSF)-PE-CF594 (BD Biosciences). Cells were analyzed with an LSR Fortessa flow cytometer (BD Biosciences), and FlowJo software was used to acquire and analyze experimental data.

Table 2. List of fluorochrome-conjugated mAbs used for ICS

Target	Fluorochrome conjugation	Source
Anti-human CD3	BUV395	BD Biosciences
Anti-human CD4	BUV496	BD Biosciences
Anti-human CD8	FITC	BD Biosciences
Anti-human CD25	APC-Cy7	BD Biosciences
Anti-human CD45RA	BV786	BD Biosciences
Anti-human CXCR3	BV421	BD Biosciences
Anti-human CCR6	BV711	BD Biosciences
Anti-human CCR7	BV510	BD Biosciences
Anti-human IL-1R1	PE	R&D Systems
Anti-human IL-17A	BV650	BD Biosciences
Anti-human IFN-γ	BV605	BD Biosciences
Anti-human GM-CSF	PE-CF594	BD Biosciences
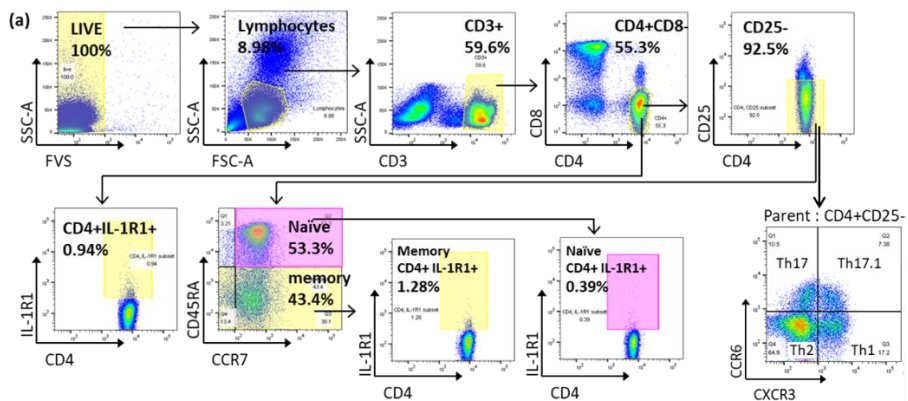
Figure 4. Gating strategy for CD4+ T cell population. Fresh or cultured cells were stained with fluorochrome-conjugated mAbs shown in Table 2 and analysed.

6. Fluorescence-activated cell sorting (FACS)

For co-culture assays, PBMC was resuspended in 500ul of cold Pre-Sort Buffer (PSB, BD Biosciences) and 1ul of fluorochrome-conjugated mAbs against CD4-BUV496 and CD56-APC (BD Biosciences) was added per 1×10^7 cells. Incubation was performed for 30 mins on ice, protected from light, and washed with PSB. Cells were prepared to a final concentration of 1×10^6 cells per 1ml PSB to acquire data by the FACS sorter (FACSAria, BD Biosciences).
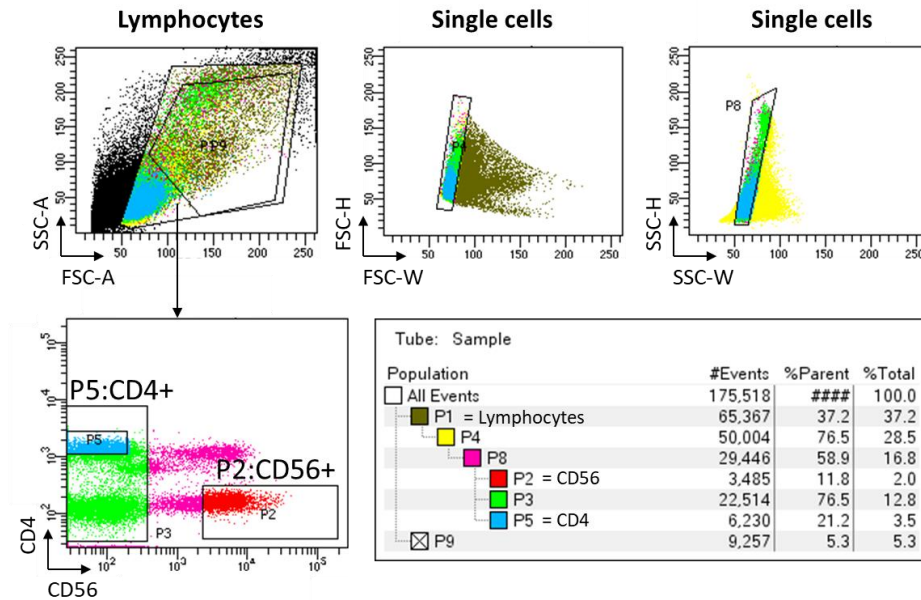
Figure 5. Flow cytometry sorting of CD4+ and CD56+ cells. Fresh PBMC were stained with fluorochrome-conjugated mAbs against CD4 and CD56 and sorted by FACSAria. Sorted CD4+ and CD56+ cells were used in subsequent co-culture assays.

7. Co-culture assay

Sorted CD4+ and CD56+ cells were incubated with the same stimulation doses as the bystander activation cell culture. The cell ratio was 1:1. 2×10^5 cells per well were cultured with IL-7, IL-1β, and IL-23 in a 96-well plate (Thermoscientific). After 3 days, cells were stained with fluorochrome-conjugated mAbs below (Table 3). Next, the cells were washed, permeabilized, and fixed with the ICS protocol. Their cytokines production was detected with IL-17A-BV650, IFN-γ-BV605, and GM-CSF-PE-CF594 (BD Biosciences).

Table 3. List of fluorochrome-conjugated mAbs used for co-culture assay

Target	Fluorochrome conjugation	Source
Anti-human CD3	BUV395	BD Biosciences
Anti-human CD4	BUV496	BD Biosciences
Anti-human CD8	FITC	BD Biosciences
Anti-human CD25	APC-Cy7	BD Biosciences
Anti-human CD45RA	BV786	BD Biosciences
Anti-human CCR7	BV510	BD Biosciences
Anti-human CD56	APC	BD Biosciences
Anti-human IL-1R1	PE	R&D Systems
Anti-human IL-17A	BV650	BD Biosciences
Anti-human IFN-γ	BV605	BD Biosciences
Anti-human GM-CSF	PE-CF594	BD Biosciences

8. Statistical analysis

The statistical significance of data was assessed using GraphPad Prism software (Graphpad version5). One-way ANOVAs with Tukey's multiple comparison tests and two-way ANOVAs with Tukey's multiple comparison test or Sidak's multiple comparison test were used for analysis, with a probability of less than 0.05 ($p < 0.05$) being considered statistically significant.

III. RESULTS

1. *Ex vivo* IL-1R1 expressions in different subtypes of CD4+ T cells.

To identify which subtypes of CD4^{+} T cell predominantly express IL-1R1, we first compared *ex vivo* IL-1R1 expression of different CD4^{+} T cell subsets in the peripheral blood of HC and MS (Fig. 6A and 6B). Among four subsets of T helper (Th) cells, based on CXCR3 and CCR6 expression, Th17 (CXCR3-CCR6+) cells and Th17.1 (CXCR3+CCR6+) cells displayed a higher expression of IL-1R1 compared with Th1 (CXCR3+CCR6-) and Th2 (CXCR3-CCR6-) cells in both MS and HC. There was no difference in *ex vivo* IL-1R1 expression between MS and HC for all subtypes of T cells.

For the comparison of IL-1R1 expression in memory (CD4+CD25-CD45RA^{-}) and naïve (CD4+CD25-CD45RA^{+}) CD4^{+} T cells between MS and HC, we utilized *ex vivo* surface staining with fresh PBMC from HC and MS patients. When comparing naïve and memory CD4^{+} T cells, a higher expression of IL-1R1 was found in memory CD4^{+} T cells for both MS and HC (Fig. 7A and 7B). Also, we confirmed that the memory type proportion is further increased in CD4+IL-1R1+ cells than in naïve type in both HC and MS patients (Fig. 7C).
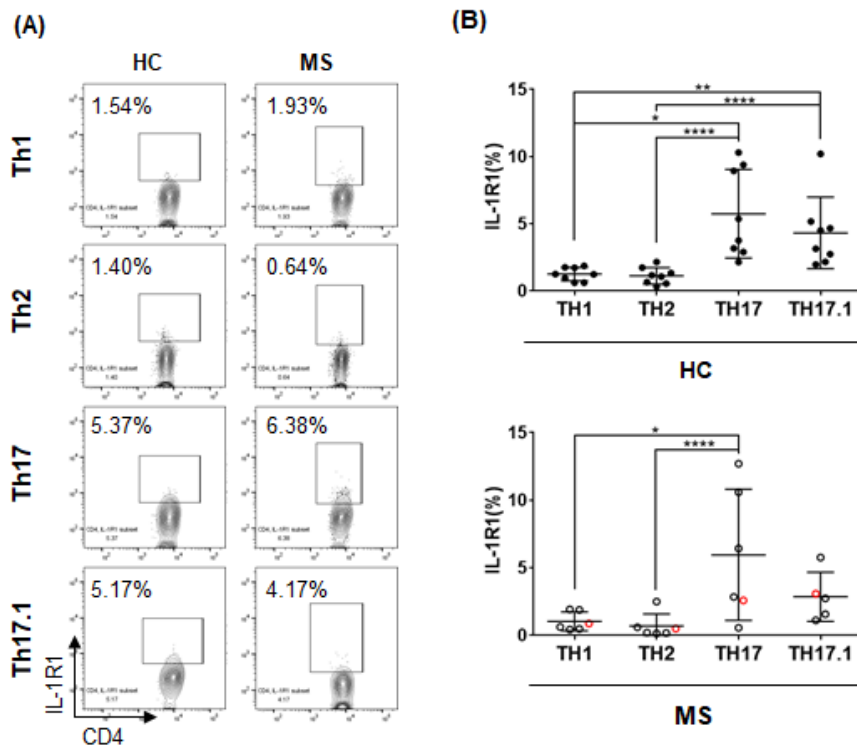
Figure 6. The *ex vivo* proportion of IL-1R1+ cells in CD4+ T cell subtypes. PBMC was isolated and stained *ex vivo* for CD4+ T cell subtyping and the expression of IL-1R1. (A) Representative flow cytometry plots and (B) the proportion of IL-1R1-expressing cells among CD4+ T cell subtypes with 8 HC and 6 MS patients (IFN-β, n=5; NTZ, n=1). Red-points represent NTZ-treated, and black-points represent IFN-β treated MS patients. Values are expressed as Mean ± SEM. Data was analyzed by one-way ANOVA. *P< 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
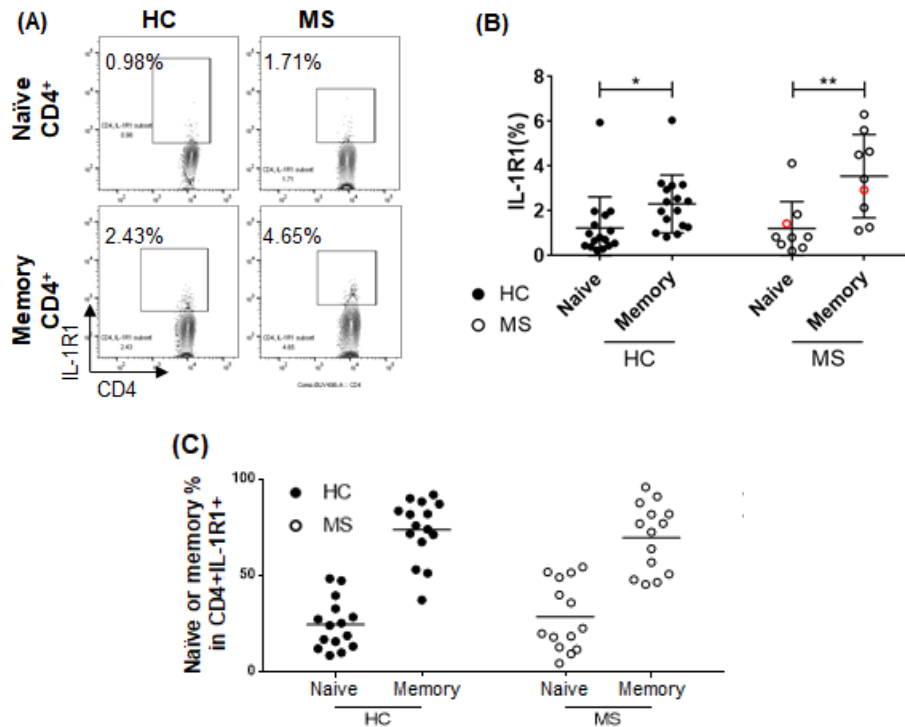
Figure 7. The proportion of IL-1R1+ cells in naïve and memory CD4+ T cells. Fresh PBMC was isolated and stained with fluorochrome-conjugated mAbs for distinguishing between naïve or memory CD4+ T cells and the expression of IL-1R1. (A) Representative flow cytometry dot plots and (B) the graph indicates IL-1R1 expressions in memory or naïve CD4+ T cells in both 16 HC and 9 MS patients (IFN-β, n=8; NTZ, n=1). (C) In 15 HC and 14 MS patients, it was confirmed that most of CD4+ IL-1R1+ cells were memory type (CD4+CD25-CD45RA-). Red-points represented NTZ-treated, and black-points represented IFN-β treated MS patients. Values are expressed as Mean ± SEM. Data were analyzed by two-way ANOVA (B and C). *P< 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

2. IL-1R1 expressions following IL-1β and IL-23 stimulation over time.

To investigate the phenotypes of CD4+IL-1R1+ cells after bystander activation, we first set up the culture conditions (Fig. 8A and 8B). The concentration of IL-7, IL-1β, and IL-23 is confirmed, according to the research about bystander-activated CD4+ T cells [77]. Sorted CD4+ T cells from HC were cultured with the bystander activation for 6 hours and then at days 1, 3 and 5. After each culture period, it was confirmed that the IL-1R1 expression in the CD4+ T cells and memory CD4+ T cells started from Day1 and increased over time. However, the expression of IL-1R1 decreased on Day 5 compared to Day 3. It was determined that the Day3 time point was optimum for the analysis of the phenotype and cytokines production for the bystander-activated CD4+ T cells. Furthermore, the frequency of IL-1R1+ cells in CD4+ or memory CD4+ T cells increased according to the bystander activation.

IL-1R1+ CD4

IL-1R1+ memory CD4

■ UNSTI   ■ IL-7   ■ IL-7+IL-1b+IL-23
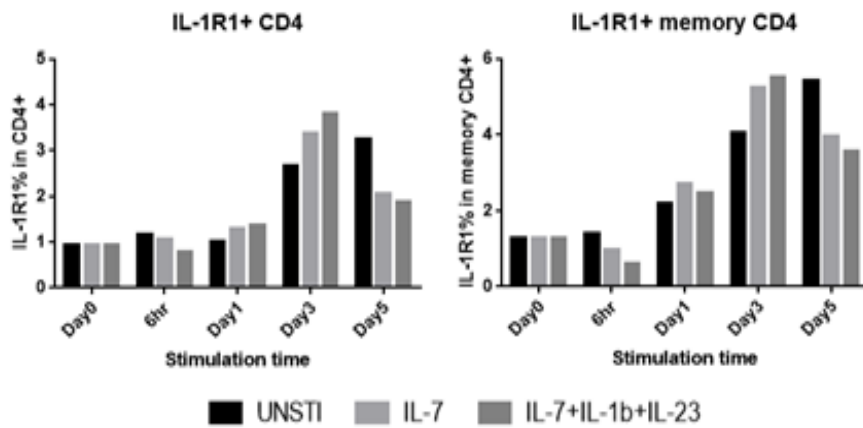
Figure 8. The expression of IL-1R1+ according to the stimulation conditions. With the IL-7, IL-1β, and IL-23 stimulations, the expression level of IL-1R1+ in total CD4 T cells or memory CD4 T cells was highest on Day 3, than the un-stimulation (UNSTI) control or IL-7 alone.

3. IL-1R1 expressions in subtypes of CD4+ T cells upon bystander activation.

Analyses were conducted in order to confirm whether the results of *ex vivo* surface staining were similar after bystander activation. After bystander-activation culture for 3 days, cells were harvested and the frequency of IL-1R1 was analyzed in helper T cell subtypes. As we previously determined, the CD4+IL-1R1+ cells increased upon bystander activated condition (Fig. 9A). Unexpectedly, the frequency of CD4+IL-1R1+ cells were also increased in IL-7 only, as in IL-7, IL-1β, and IL-23 stimulation conditions. CD4+IL-1R1+ cells were highest within Th17.1 cells compared to other Th1, Th2, and Th17 in both HC and MS patients, as in *ex vivo* surface staining (Fig. 9B). In HC, the level of CD4+IL-1R1+ cells was also greater in Th17 than Th1 and Th2.

The major subtype of CD4+IL-1R1+ cells among the CD4+ T cells in *ex vivo* surface staining was identified as the memory type. In both HC and MS patients, the majority of CD4+IL-1R1+ T cells were memory type in all simulations (Fig. 10A). In HCs and patients with MS, the number of IL-1R1+ cells increased after either IL-7 alone or bystander activation compared to unstimulated (Fig. 10B and 10C). There was no statistical difference in the frequency of CD4+IL-1R1+ and memory CD4+IL-1R1+ T cells between HC and MS patients upon bystander activation (Fig. 10B). Similarly, the differences in IL-1R1 expression in both CD4+ and memory CD4+ cells after MS treatment showed no statistical significance after bystander activation.
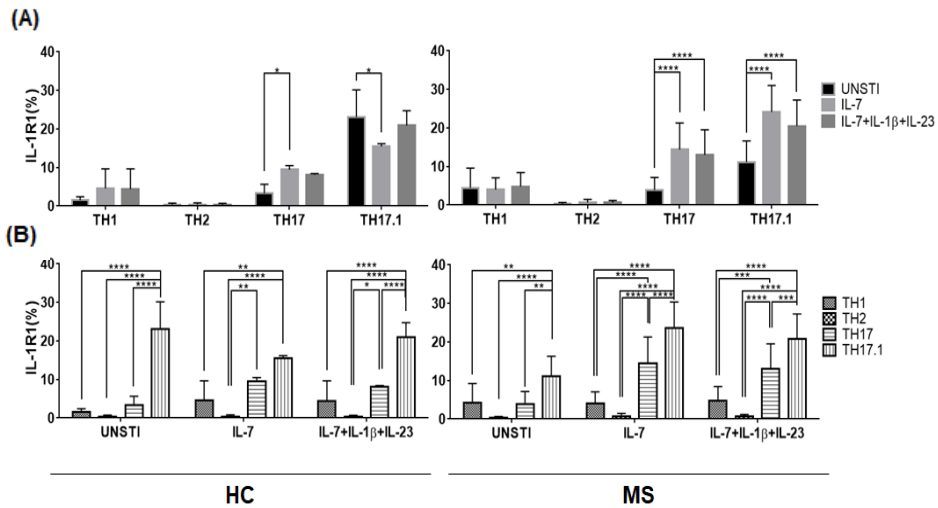
Figure 9. The frequency of IL-1R1+ cells in the subtypes of CD4+ T cells. Isolated CD4+ T cells were cultured with IL-7, IL-1β, and IL-23 for 3 days. Cells were harvested and stained for CD4+ T cell subtyping and the expression of IL-1R1 by ICS. (A) The graphs show the proportion of IL-1R1-expressing cells was highest in Th17.1 among CD4+ T cell subtypes, in both HC and MS patients. (B) The graph indicates the IL-1R1 expressions in CD4+ T cells increased after bystander activation (IL-7, IL-1β, and IL-23) in both HC and MS patients. Values are expressed as Mean ± SEM. Data were analyzed by two-way ANOVA. *P< 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
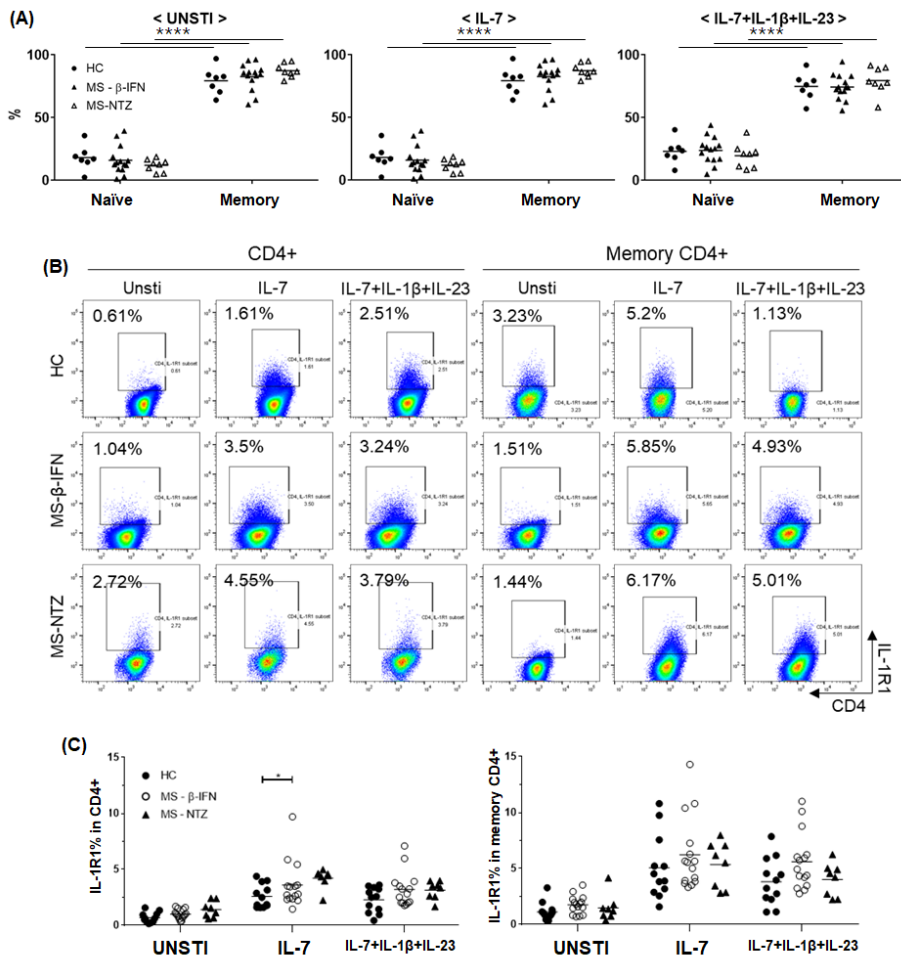
Figure 10. The frequency of IL-1R1+ cells in CD4+ T cells. CD4+ T cells were stimulated with IL-7, IL-1β, and IL-23 for 3 days and analyzed by ICS. (A) Comparison of naïve and memory type cells in CD4+IL-1R1+ cells. (B) Representative dot plot and (C) the graphs showed CD4+IL-1R1+ and memory CD4+IL-1R1+ T cells had no difference between HC and MS patients, despite MS treatment.

4. IL-1β and IL-23 promoted higher IL-17A production from memory CD4+IL-1R1+ in MS compared with HC.

To evaluate the contribution of proinflammatory cytokine-producing bystander-activated CD4+ T cells in MS, we next examined the frequency of IL-17A, IFN-γ, and GM-CSF-producing CD4+IL-1R1+ T cells post bystander activation. Since the previous experiment confirmed that bystander-activated CD4+ T cells are memory type, it was theorized that cytokine production focuses on memory CD4+IL-1R1+ cells. In response to IL-1β and IL-23, the production of IL-17A and GM-CSF was increased in memory CD4+IL-1R1+ T cells, whereas there were no changes in IFN-γ production (Fig. 11A-C). In HC, such an increase was only seen in IL-17A, but not for IFN-γ and GM-CSF production. Similar to memory CD4+IL-1R1+ T cells, patients with MS showed a significantly higher frequency of IL-17A-producing IL-1R1+ Th17 and Th17.1 cells compared with HC.
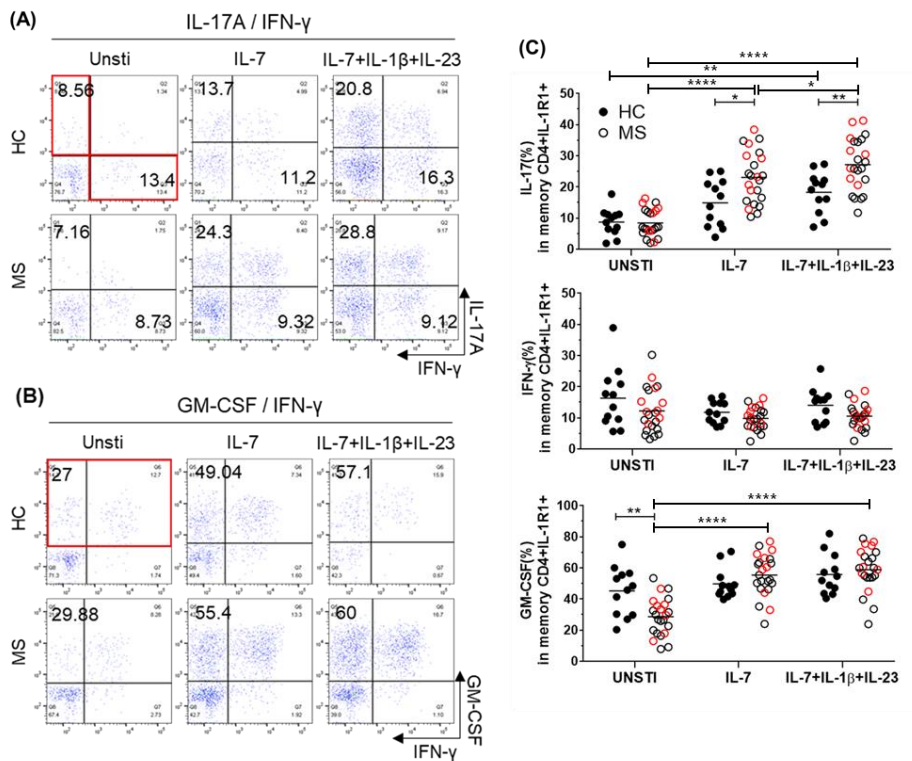
Figure 11. The production of IL-17A is increased in memory CD4+IL-1R1+ cells upon bystander activation in MS patients. (A, B) Representative data and (C) Graphs show the frequency of IL-17A producing cells, not IFN-γ or GM-CSF in memory CD4+ IL-1R1+ increased in all 23 MS patients (IFN-β, n=15; NTZ, n=8), compared to HC (n=12), under the of IL-7, IL-1β and IL-23 combination condition. Red-circle represent NTZ-treated, and black-filled dots represent IFN-β treated MS patients. All experiments used a method of incubating sorted CD4 T cells under each stimulation condition for 3 days. Data are presented as the Mean ± SEM; *P< 0.05, and **P < 0.01.

5. There was no difference in IL-17A producing cell frequency in MS patients compared to HCs in total PBMC.

Additionally, we investigated the difference in phenotypes and cytokine production in IL-1R1+ cells by stimulating the PBMC upon the same bystander activation, IL-7, IL-1β, and IL-23. The PBMC experiments were performed to evaluate the contribution of bystander-activated CD4+ T cells in the peripheral environment as well as in sorted CD4+ T cells. The PBMC of 12 HC and 22 patients with MS (IFN-β, n=15 and NTZ, n=7) were used for the PBMC culture. We identified the majority of CD4+IL-1R1+ cells were memory type, however, there was no difference between helper T cell subtypes in the PBMC. CD4+IL-1R1+ cells were memory type in unstimulated, IL-7 only, and IL-7, IL-1β, and IL-23 conditions (Fig. 12A). When the expression of IL-1R1 in helper T cells was investigated, it was revealed there was no difference on IL-1R1+ T cells in both HC and MS patients in every subtype (Fig. 12B). The frequency of memory CD4+IL-1R1+ cells according to stimulation was confirmed (data not shown).

With regard to pro-inflammatory cytokines producing cells in memory CD4+IL-1R1+ T cells in PBMC, the frequency in all groups remained the same in regardless of stimulations (Fig. 12C-E). As bystander-activated CD4+ T cells had previously been shown to be a memory type, the production of cytokines were focused on memory CD4+IL-1R1+ cells. The results showed that when PBMC was used, there was no difference in IL-17A and GM-CSF producing cell frequencies in HCs and MS patients in memory CD4+IL-1R1+ cells

(Fig. 12C-E), contrary to sorted CD4+ T cells. Like the sorted CD4+ T cells, IFN-γ producing cells were nor different compared to MS and HC (Fig. 12D and 12E). Collectively, in PBMC settings, bystander activation did not induce the expression of IL-1R1 and the production of IL-17A in memory CD4+IL-1R1+ T cells.
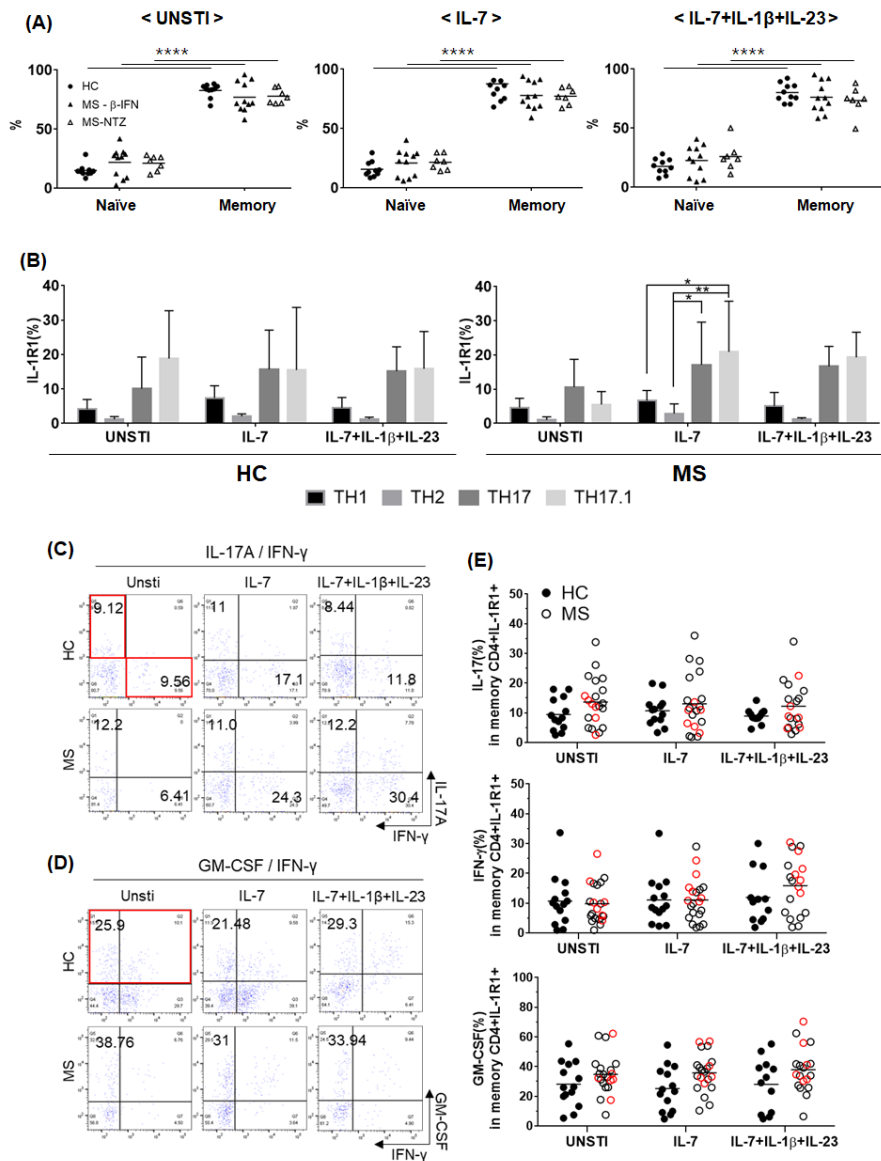
Figure 12. PBMC showed no difference in IL-17A producing cell frequency in IL-1R1+ CD4+ T cells. (A) Memory type cells were higher than naïve type cells in IL-1R1-expressing cells between HC (n=10) and 16 MS patients (IFN-β, n=11 and NTZ, n=5). (B) There were no statistical differences between helper T cell subtypes about IL-1R1+ expression. (C, D) Representative dot plot data explains the

representative figures of IL-17, IFN-γ, and GM-CSF producing cell frequencies in memory CD4+IL-1R1+ T cells upon bystander activation. The experiments were performed with HC (n=16) and, 22 patients with MS (IFN-β, n=15 and NTZ, n=7). (E) IL-17, IFN-γ, and GM-CSF producing cell frequencies showed no difference between HC and MS patients. All experiments used a method in which incubate fresh PBMCs were incubated under each stimulation condition for 3 days. Red-circle represent NTZ-treated, and black-filled dots represent IFN-β treated MS patients. Mean ± SEM.

6. CD56+ NK cells may regulate IL-17A production from bystander T cell activation.

To investigate interactions between CD4+ T cells and other lymphocytes upon bystander activation, we evaluated the stimulatory effect of IL-1β and IL-23 on CD4+ T cells cultured *in vitro* with PBMC. Unlike the findings of *in vitro* activation of CD4+ T cells, we found neither increased expression of IL-1R1 nor enhanced the production of IL-17 from CD4+IL-1R1+ memory T cells in PBMC in the presence of IL-1β and IL-23. Given that bystander CD4+ cell activation was inhibited in PBMC compared to isolated CD4+ T cells, we hypothesized that other immune cells could regulate the activation of bystander T cells. Among the many candidates in the PBMC, we thought CD56+ cells could attenuate this phenomenon as both CD19+ cells and CD56-CD19-CD8- cells failed to results in a significant decrease of IL-17A production after co-culture of each cell type and CD4+ cells after bystander activation in our pilot study (Fig. 13A). Furthermore, previous studies reported that CD56+ NK cells can suppress pathogenic autoimmune Th17 cell activity [23, 24] and dysregulation of impairment of NK cells has been linked to MS disease activity [25, 26]. To investigate whether the NK cells suppress the IL-17 production from CD4+IL-1R1+ memory T cells, we isolated and activated CD4+ cells and CD56+ NK cells from 6 HCs with IL-1β and IL-23 in co-culture at a 1:1 ratio (CD4:CD56 cells). Upon stimulation, the level of IL-1R1 expression on CD4+ memory T cells were decreased with the presence of CD56+ NK cells. However, the frequency of IL-17 producing CD4+IL-1R1+ memory T cells decreased in the presence of CD56+ NK cells (Figure 14A and

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14B). With IFN-γ and GM-CSF production, there was no significant change due to CD56+ cells (Fig. 13B). This data suggests that CD56+ NK cells may play a key role in regulating the production of IL-17 by activation of bystander T cells.
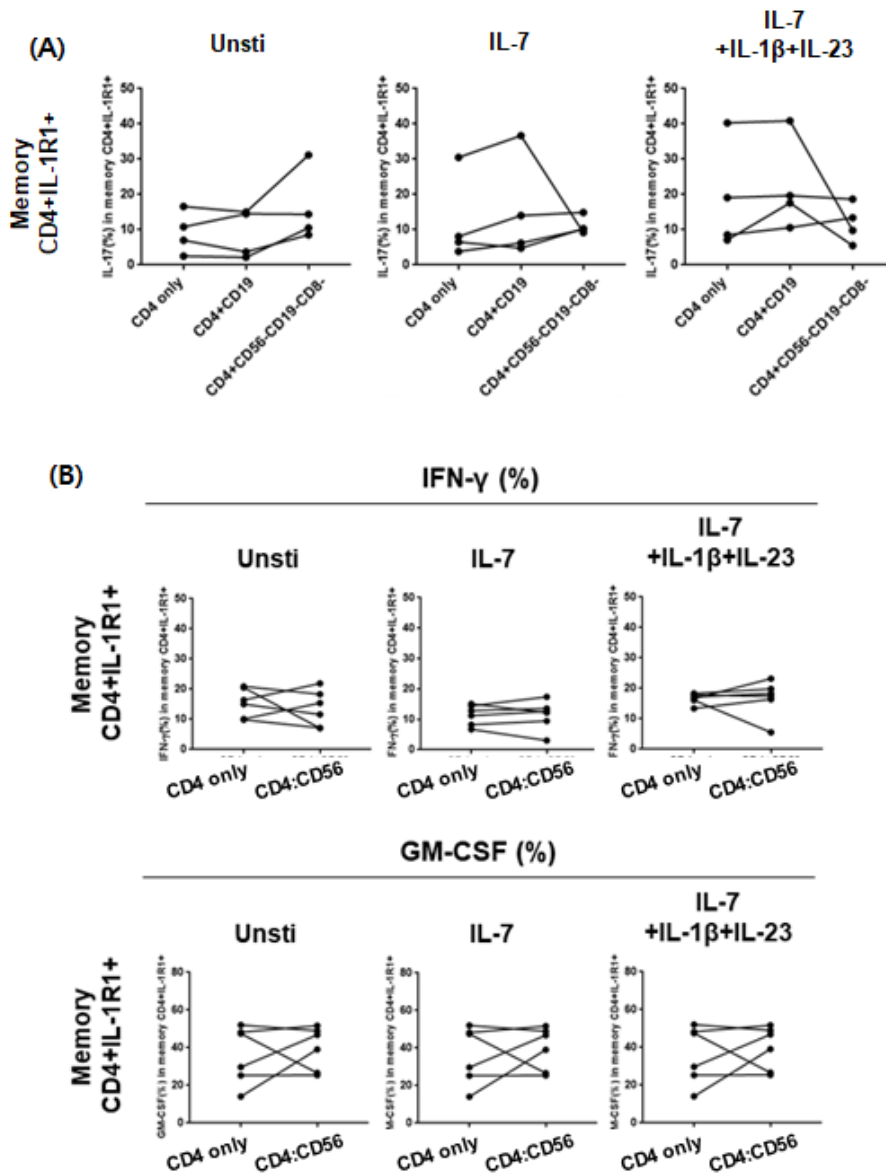
Figure 13. Pro-inflammatory cytokines after coculture experiments.
(A) IL-17A producing cells had no difference between CD4+ only, CD19+, and CD56-CD19-CD8- cells. (B) There is no difference in percentages of IFN-γ, and GM-CSF producing cells in memory CD4+IL-1R1+ cells after CD4+ cells co-cultured with CD56+. CD56+ cells were

seeded with CD4+ cells in a 1:1 ratio from 4 HCs, CD19+, and CD56-CD19-CD8- cells were sorted from 4HCs. The cells were incubated with IL-7 or IL-7+IL-1β+IL-23 for 3 days.

(A)

IL-1R1(%)

(B)

IL-17A-producing cells(%)
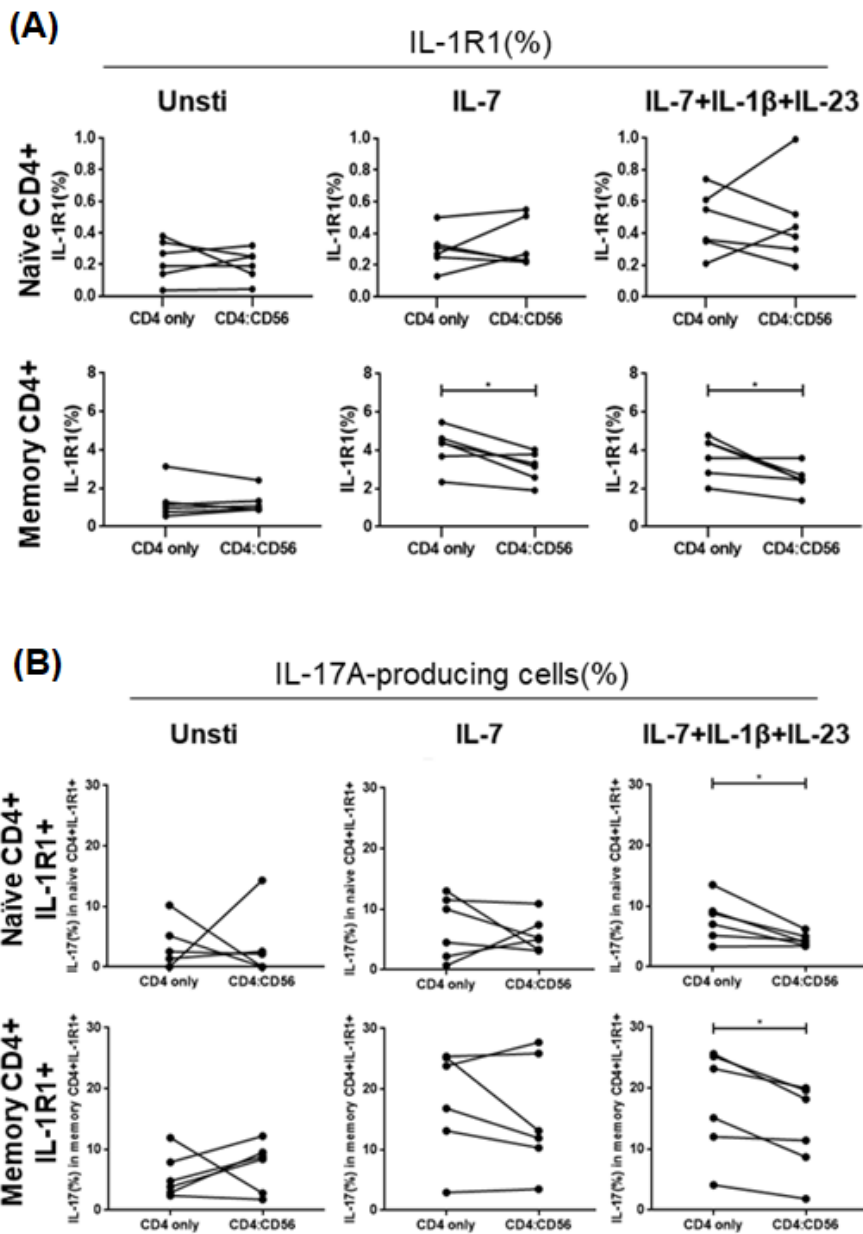
Figure 14. CD56+ cells may attenuate the IL-17A producing cell activity of memory CD4+IL-1R1+ T cells. (A) IL-1R1 expression decreased in memory CD4+ T cells after co-culture CD4+ cells with

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CD56+ cells. (B) Unsurprisingly, in naïve and memory CD4+ IL-1R1+ cells, the frequency of IL-17A producing cells decreased in the CD4:CD56 group upon bystander activation. All experiments used sorted cells from HC and cultured with appropriate conditions for 3 days. *p < 0.05.

IV. DISCUSSION

Although our understanding of the importance of bystander-activated CD4+ T cells in various autoimmune diseases is improving, many of the studies carried out on the implications of these cells have been conducted on EAE, and little research exists on patients with MS. Here, we showed that the frequency of CD4+ IL-1Rl+ T cells was significantly higher in memory and Th17/Th17.1 cells than in naïve and Th1/Th2 cells. These results are in accordance with previous studies that have shown higher IL-1Rl+ expression in human memory cells over naïve CD4+ T cells at the gene level [77, 82] as well as in positive cell frequency [77]. Likewise, in vitro-differentiated Th17 cells in the presence of TCR stimulation expressed higher levels of IL-1R1 gene expression than Th1- or Th2-differentiated cells [77, 82]. We further demonstrated that IL-1Rl expression increased following IL-1β and IL-23 stimulation and in the absence of TCR stimulation in memory and Th17/Th17.1 cells. On the other hand, such an increase was not observed in naïve and Th1/Th2 cells, with the exception of MS naïve CD4+ T cells. Intriguingly, IL-1β and IL-23 markedly amplified the IL-17A and GM-CSF production from CD4+IL-1R1+ memory and Th17/Th17.1 cells. Furthermore, it was observed that this increased production of IL-17A from these subtypes of CD4+ T cells was significantly higher in MS compared with the HC. Given the well-known pathogenic role of IL-17 in MS, these results imply a potential contribution of IL-17A producing bystander-activated CD4+ T cells to the disease process of MS.

In this study, the addition of IL-1β and IL-23 was used to create a bystander-activated environment. IL-1β is one of the essential factors in

the differentiation of Th17 cells and a potent cytokine inducer for Th17 cells in the absence of TCR engagement [83]. IL-23 is also essential for the generation of pathogenic Th17 cells [52]. However, IL-23 alone cannot induce bystander activation in memory CD4+ T cells [84], whereas, in combination with IL-1β, their synergistic effect increases the production of pro-inflammatory cytokines in Th17 and memory CD4+ T cells in a TCR-independent environment [77, 84].

An increase in IL-1R1 gene expression in CD4+ T cells derived from MS in comparison to HC has been previously reported [82], whereas, in this study, there were no significant differences in the frequency of CD4+IL-1R1+ between MS and HC, both *ex vivo* and after *in vitro* culture with IL-1β and IL-23. This discrepancy may have been due to differences between gene and protein expression. However, it is possible that treatment with disease-modifying therapy (either IFN-β or natalizumab) may affect the frequency of CD4+IL-1R1+ cells. Likewise, the insignificant difference between MS and HC in the production of GM-CSF following bystander activation may also be a result of treatment. Thus, this was considered to be the main limitation of the study, and therefore further studies using patients with MS who are treatment-naïve or in exacerbation are required. Despite the potential treatment effect, it is of note that the IL-17A production following bystander activation was significantly higher in memory and IL-1R1+ Th17/Th17.1 CD4+IL-1R1+ cells derived from MS compared with HC.

The production of IL-17A in memory CD4+IL-1R1+ cells was reduced upon bystander activation when PBMC was used compared to CD4+ T cells. Among the many immune cells in PBMC, CD56+ NK cells are

well known as a regulator for autoimmune disease. In particular in MS, it was demonstrated that NK cells can mediate T cell responses in various cytotoxic processes through apoptotic pathways [85, 86]. They have also been shown to suppress the function of CD4+ Th17 in the CNS [87]. Recent research found that immune regulation by NK cells was associated with the reduction of innate-like T cells in MS after autologous hematopoietic stem cell transplantation [88]. Therefore, we anticipate that one of the factors influencing IL-17A production of memory CD4+IL-1R1+ cells in bystander activation was CD56+ NK cells. The co-culture experiments were performed in an attempt to determine the reasons for the difference in the frequency of IL-17A producing CD4+IL-1R1+ T cells between PBMC and sorted CD4+ T cells. As expected, our results revealed that CD56+ cells may mediate IL-17A producing cells in memory CD4+IL-1R1+ T cells following bystander activation.

It is necessary to analyze the relationship between memory CD4+IL-1R1+ cells and other immune cells. In our NK cell regulation experiment, the cells that affect IL-17A production of memory CD4+IL-1R1+ cells in bystander activation may exist in other immune cells besides CD56+ cells. Lymphocytes with regulatory functions in the immune system could be candidates. For example, Tregs (regulatory T cells), which traditionally suppresses abnormal Th1 and Th17 responses [89, 90] were revealed to regulate ILCs [91]. Some Tregs recently identified that they can be activated in an antigen-independent environment [92]. It is possible that the function of the bystander-activated CD4+ T cell may be reduced by Bregs (regulatory B cells) because they inhibit the CD4+ T cell proliferation and IL-17 production [93, 94]. Among Bregs, the B-1a

cells create an innate response and emit immune-suppressive cytokines [95, 96]. In addition, other immune cells with the regulatory functions are able to regulate the IL-17A production of memory CD4+IL-1R1+ cells more reliably than CD56+ cells. It is also necessary to investigate whether the results are replicable in a variety of treated MS patients as only HCs were used in this experiment. Additionally, better understanding of the precise role of memory CD4+IL-1R1+ cells and their process in the innate/adaptive immune system is required to determine how they contribute to disease. A molecular-level mechanism study that induces bystander activation to memory CD4+IL-1R1+ cells in autoimmune diseases, including MS, should be undertaken. These studies will provide a broader perspective and improve our understanding of the pathological causes and phenomena of autoimmune diseases.

V. CONCLUSION

In summary, the results of this study suggest the potential contribution of IL-17A-producing bystander-activated CD4+ IL-1R1+ T cells in MS. Further studies to decipher the precise role of CD4+IL-1R1+ cells and their process in the innate and adaptive immune system, as well as their contribution to disease are warranted.

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ABSTRACT (IN KOREAN)

다발성 경화증에서 방관자 활성 CD4+IL-1R1+ 세포에 의한 IL-17A 생성

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김소연

다발성 경화증은 자가 항원을 인식하는 CD4+ T 세포에 의해 발생하는 자가면역 탈 수초 질환으로 알려져 있다. 그러나 다발성 경화증의 동물모델인 자가면역성 뇌척수막염 동물실험 등의 연구에서 전 염증성 cytokines인 IL-1β와 IL-23 (방관자 활성화)에 의해 CD4+ T 세포가 항원 독립적으로 활성화되면 IL-1R1이 발현되며, 염증성 cytokine인 IL-17을 방출함으로써 다발성 경화증의 악화에 기여할 수 있다는 가능성이 제기되었다. 따라서, 본 연구에서는 다발성 경화증 환자에서 방관자 활성 CD4+ T 세포의 빈도와 기능에 대해 연구하였다. CD4+IL-1R1+ T 세포는 방관자 활성화 후에도, 기억 (memory)와 Th17/Th17.1 세포에서 현저하게 증가하였으며, IL-17A 생성 또한 대조군에 비해 다발성 경화증 환자에서 유의하게 증가함이 밝혀졌다. 그러나 말초 혈액 단핵세포를 방관자 활성화시켰을 때는 다발성 경화증 환자에서 IL-17A의 증가가 나타나지 않았으며, 그 이유 중 하나로 자연살상세포에 의한 CD4+ T cell의 활성 저하를 제시하였다. 해당 연구는 CD4+IL-1Rl+ T 세포가 항원 독립적으로 IL-17A를 생산함으로써 다발성 경화증에서 잠재적인 역할을 하고 있음을 보여주었다.

핵심 되는 말: 자가면역질환, 다발성 경화증, 방관자 활성화, IL-17A