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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 V $\alpha$ 7.2-J $\alpha$ 33 T-cell antigen receptor  $\alpha$  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<sup>+</sup> invariant NKT cells and of CD56<sup>bright</sup> 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- $\gamma$  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<sub>H</sub>1 cells.

**Keywords:** CD161, immune regulation, IFN- $\gamma$ , 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- $\gamma$ -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- $\gamma$  (T<sub>H</sub>1) and/or IL-17 (T<sub>H</sub>17) 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<sub>H</sub>1 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<sup>+</sup> invariant natural killer T (iNKT) cells was reported to be biased toward T<sub>H</sub>2, 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<sup>+</sup>CD25<sup>high</sup> 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)  $\alpha$  chain distinct from V $\alpha$ 24 iNKT cells (10, 11). MAIT cells express a canonical V $\alpha$ 7.2-J $\alpha$ 33 TCR  $\alpha$  chain in preferential combination with V $\beta$ 2 and V $\beta$ 13 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<sup>+</sup>iNKT cells and CD56<sup>bright</sup> 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- $\gamma$  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:  $40.2 \pm 1.7$  (mean  $\pm$  SE)] and 16 age- and sex-matched healthy controls (HC) (age:  $40.4 \pm 2.6$ ) 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:  $40.7 \pm 2.0$ ) were in remission and 7 (age:  $38.1 \pm 3.2$ ) were in an acute phase of relapse. None of the patients in remission was taking any medications affecting the immune system, such as IFN- $\beta$ , 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- $\gamma\delta$  mAb, FITC-anti-TCR-V $\alpha$ 24 mAb, phycoerythrin (PE)-anti-CD161 mAb, PE-anti-TCR-V $\beta$ 11 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-CD8 $\alpha$  mAb, APC-anti-CCR 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 ( $5 \times 10^5$  cells per well in 24-well culture plates) were stimulated with 50 ng ml<sup>-1</sup> phorbol-myristate-acetate (PMA) (Sigma, St Louis, MO, USA) and 500 ng ml<sup>-1</sup> ionomycin (IM) (Sigma) for 4 h at 37°C in 5% CO<sub>2</sub>. Monensin (BD Bioscience) was added in the last 2 h of culture at a concentration of 2  $\mu$ M. 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- $\gamma$  (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<sup>+</sup>CD19<sup>-</sup>TCR- $\gamma\delta$ <sup>-</sup>CD161<sup>high</sup>3C10<sup>+</sup>) or other T cells (CD5<sup>+</sup>CD19<sup>-</sup>TCR- $\gamma\delta$ <sup>-</sup>CD161<sup>-</sup>3C10<sup>-</sup>) were isolated from the PBMC of HC using a FACS Aria cell sorter and cultured in 96-well flat-bottom plates at  $1 \times 10^5$  cells per well with RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.05 mM 2-mercaptoethanol, 100 U ml<sup>-1</sup> penicillin and 100  $\mu$ g ml<sup>-1</sup> streptomycin. Cells were stimulated with immobilized anti-CD3 mAb (5  $\mu$ g ml<sup>-1</sup>, clone OKT3; American Type Culture Collection, Manassas, VA, USA) and soluble anti-CD28 mAb (1  $\mu$ g ml<sup>-1</sup>, clone CD28.2; Beckman-Coulter) or PMA (50 ng ml<sup>-1</sup>) and IM (500 ng ml<sup>-1</sup>) for 48 h. IFN- $\gamma$ , 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<sup>+</sup>CD19<sup>-</sup>TCR $\gamma\delta$ <sup>-</sup>CD161<sup>high</sup>3C10<sup>+</sup> 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  $2 \times 10^5$  cells per well and stimulated with 2  $\mu$ g ml<sup>-1</sup> 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 ( $1 \times 10^5$  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  $\mu$ g ml<sup>-1</sup>), anti-inducible co-stimulator (ICOS)-ligand (L) mAb (10  $\mu$ g ml<sup>-1</sup>) (both from eBioscience), anti-transforming growth factor (TGF)- $\beta$  mAb (10  $\mu$ g ml<sup>-1</sup>; 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

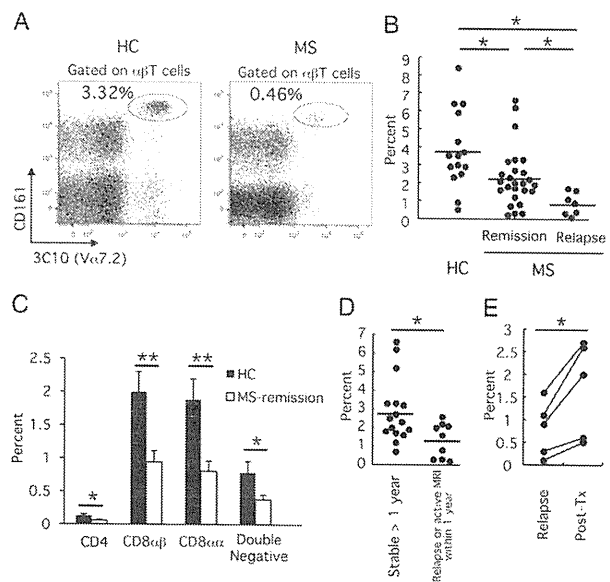
Total RNA was extracted from isolated MAIT cells or control T cells using RNeasy spin columns (QIAGEN, Germantown, MD, USA) and reverse transcribed into complementary DNA using Primescript reverse transcriptase (Takara, Ohtsu, Japan). Levels of IFN- $\gamma$  and IL-17 mRNA were measured by quantitative PCR using an SYBR Premix Ex Taq Kit (Takara) on a LightCycler1.5 (Roche, Basel, Switzerland). Expression levels relative to those of  $\beta$ -actin are presented. The primer pairs used were as follows: IFN- $\gamma$  forward, 5'-ACAGG-GAAGCGAAAAAGGAGTCAG-3' and IFN- $\gamma$  reverse, 5'-CATGGGATCTTGCTAGGTTGG-3'; IL-17 forward, 5'-CCAG-GATGCCCAAATTCTGAGGAC-3' and IL-17 reverse, 5'-CAA GGTGAGGTGGATCGGTTGTAG-3' and  $\beta$ -actin forward, 5'-CACTCTCCAGCCTTCCTCC-3' and  $\beta$ -actin reverse, 5'-GCGTACAGGTCTTTGCGGATG-3'.

### Results

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

Previously, CD161<sup>high</sup> 3C10 (V $\alpha$ 7.2)-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 $\alpha$ 7.2<sup>+</sup>CD161<sup>high</sup> 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  $\alpha\beta$ T cells was  $3.79 \pm 0.52\%$  (mean  $\pm$  SEM). In MS remission, the frequency of MAIT cells was  $2.33 \pm 0.39\%$ , which was significantly lower than that in HC (Fig. 1B). The frequencies of V $\alpha$ 7.2<sup>+</sup>CD161<sup>low</sup> and V $\alpha$ 7.2<sup>+</sup>CD161<sup>-</sup> or V $\alpha$ 7.2<sup>-</sup>CD161<sup>high</sup> 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 V $\alpha$ 7.2 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, CD8 $\alpha\alpha$  and CD8 $\alpha\beta$  populations (13). Since all the CD4, CD8 $\alpha\beta$ , CD8 $\alpha\alpha$  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  $\alpha\beta$ T cells among PBMC was not different between HC, MS remission and MS relapse ( $61.7 \pm 4.7$ ,  $65.8 \pm 1.9$  and  $67.85 \pm 3.2\%$ , respectively).

The decrease in the frequency of MAIT cells was more profound in MS relapse ( $0.87 \pm 0.24\%$ ) (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

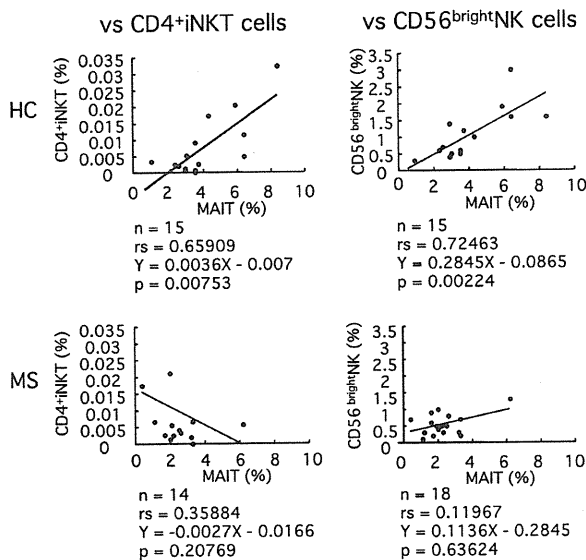


**Fig. 1.** Frequency of MAIT cells among  $\alpha\beta$ T cells in peripheral blood. (A) Representative flow cytometry profiles of CD3<sup>+</sup>TCR- $\gamma\delta$ <sup>-</sup> cells in a HC (left) and in an MS relapse (right). (B) Frequency of MAIT cells among  $\alpha\beta$ T cells in HC ( $N = 16$ ), 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, CD8 $\alpha\beta$ , CD8 $\alpha\alpha$  and DN MAIT cell sub-population among total  $\alpha\beta$ T 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<sup>+</sup>iNKT cells and CD56<sup>bright</sup> NK cells are lost in MS.

Since several other innate lymphocytes such as CD4<sup>+</sup>iNKT cells and CD56<sup>bright</sup> 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<sup>+</sup>iNKT cells and CD56<sup>bright</sup> NK cells were observed in HC. In MS patients, however, the frequency of CD56<sup>bright</sup> NK cells was decreased along with MAIT cells (Fig. 2, lower right panel). In the case of CD4<sup>+</sup>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<sup>+</sup>iNKT cells and CD56<sup>bright</sup> NK cells. The frequency of MAIT cells was plotted against the frequency of CD4<sup>+</sup>iNKT cells (CD3<sup>+</sup>CD4<sup>+</sup>TCR- $\alpha$ 24<sup>+</sup>-V $\beta$ 11<sup>+</sup>) among total T cells (left panels) or that of CD56<sup>bright</sup> NK cells (CD3<sup>-</sup>CD56<sup>bright</sup>) 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- $\gamma$  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- $\gamma$  in response to PMA and IM (Fig. 3C, upper panels). However, interestingly, the proportion of IFN- $\gamma$ <sup>+</sup> 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- $\gamma$  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<sup>+</sup> and IL-10<sup>+</sup> 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- $\gamma$  and IL-17 in MAIT cells were

not different from control T cells, and the values were comparable between HC and MS patients.

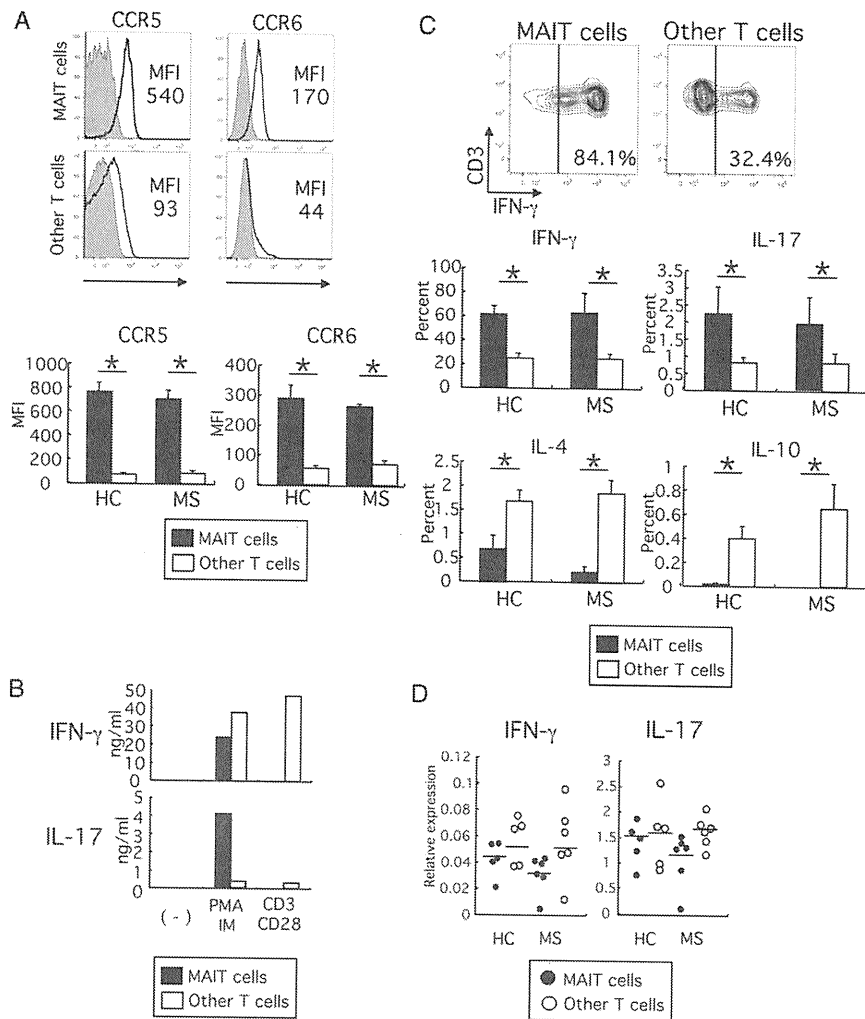
#### MAIT cells suppress IFN- $\gamma$ production from T cells in a cell contact-dependent manner

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- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$  production from T cells in peripheral blood. This IFN- $\gamma$  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- $\gamma$  suppression by MAIT cells, we first examined the involvement of suppressive cytokines such as IL-10 and TGF- $\beta$  by adding their specific mAbs to the culture. MAIT cell-mediated suppression of IFN- $\gamma$  production was not altered in the presence of these mAbs (Fig. 4C). We next examined whether MAIT cell-mediated IFN- $\gamma$  production requires cell contact. As shown in Fig. 4(B), the IFN- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$  production. These findings indicate that MAIT cell-mediated suppression of IFN- $\gamma$  production from T cells in peripheral blood required cell contact but not IL-10, TGF- $\beta$ , ICOS or B cells.

#### Discussion

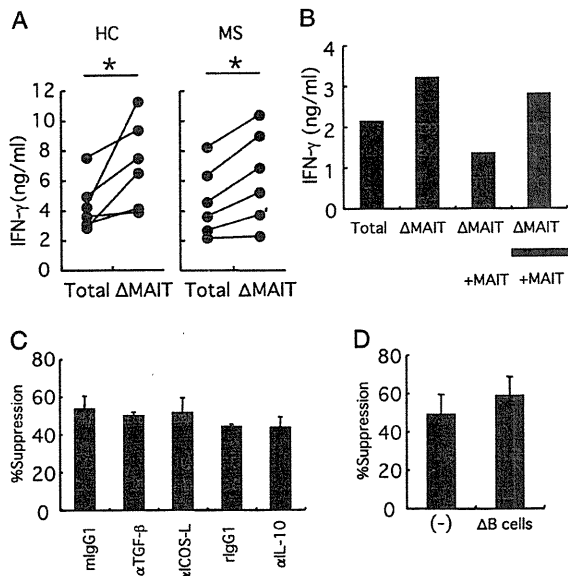
In this study, we show that MAIT cells, which comprise a large cell population in human peripheral blood, are reduced in MS patients, especially in those with active disease. Although the precise mechanism of this reduction of MAIT cells in the peripheral blood of MS patients could not be addressed in 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 ( $CD3^+TCR-\gamma\delta^-CD161^{hi}3C10^+$ ) and other T cells ( $CD3^+TCR-\gamma\delta^-3C10^-$ ) 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- $\gamma$  (upper panel) and IL-17 (lower panel) production from isolated MAIT cells ( $CD5^+CD19^-TCR-\gamma\delta^-CD161^{hi}3C10^+$ ) and other T cells ( $CD5^+CD19^-TCR-\gamma\delta^-CD161^-3C10^-$ ) 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 ( $CD3^+TCR-\gamma\delta^-CD161^{hi}3C10^+$ ) and other T cells ( $CD3^+TCR-\gamma\delta^-3C10^-$ ). Representative staining profiles of IFN- $\gamma$  from an MS remission are shown (upper panels), and results of IFN- $\gamma$ , 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- $\gamma$  (left) and IL-17 (right) mRNA expression in MAIT cells ( $CD3^+TCR-\gamma\delta^-CD161^{hi}3C10^+$ ) and other T cells ( $CD3^+TCR-\gamma\delta^-3C10^-$ ) 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  $\alpha 4\beta 1$  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- $\gamma$  production from T cells by MAIT cells. (A) IFN- $\gamma$  production from PBMC stimulated with PHA with ( $\Delta$ MAIT) or without (total) depletion of MAIT cells in HC and MS remission ( $n = 6$  each). \* $P < 0.05$  (Wilcoxon  $t$ -test). (B) IFN- $\gamma$  production from MAIT cell-depleted PBMC ( $\Delta$ MAIT) 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- $\gamma$  suppression (%suppression) by MAIT cells in the presence of mAbs against TGF- $\beta$ , ICOS-L, IL-10 (C) or when B cells were depleted from the culture ( $\Delta$ B cells) (D). Mean  $\pm$  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<sup>+</sup>iNKT and CD56<sup>bright</sup> 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<sup>bright</sup> 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<sup>+</sup>CD161<sup>high</sup> 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<sup>+</sup>CD161<sup>high</sup> T cells express V $\alpha$ 7.2 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- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$  production from T cells. We hypothesize that MAIT cells act as a sensor for environmental changes by responding to alterations in gut microflora by modulating the host's immune system. This property of MAIT cells should be favorable for host defense in most cases but may be disadvantageous in some case including MS. It is possible, however, that a novel treatment for MS might be established by enhancing the immunosuppressive property of MAIT cells through modulation of the host's gut microflora.

### Supplementary data

Supplementary data are available at *International Immunology* Online.

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# GRAIL (Gene Related to Anergy in Lymphocytes) Regulates Cytoskeletal Reorganization through Ubiquitination and Degradation of Arp2/3 Subunit 5 and Coronin 1A<sup>\*§</sup>

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

The regulation of T cell activation ensures efficient elimination of pathogens, as well as the maintenance of tolerance to self. Peripheral tolerance prevents the expansion of self-reactive T cells that escaped thymic selection, thus avoiding autoimmunity. T cell anergy is one form of peripheral tolerance that results in nonresponsiveness to antigen rechallenge following an initial partial activation; partial initial activation may result from the stimulation of T cell receptor (TCR)<sup>2</sup> in the absence of co-stimulation or the stimulation of T cells with the calcium ionophore ionomycin (1, 2). The induction of T cell anergy is inhibited by the addition of cyclohexamide, suggesting that anergy induction requires new protein synthesis (3). Recent reports have demonstrated that the induction of E3 ubiquitin ligases, including CBL-b, Itch, Deltex-1, and GRAIL (gene

related to anergy in lymphocytes), is required to induce and maintain T cell anergy (4–8). In particular, it is well known that Cbl and Cbl-b act as negative regulators of TCR or CD28 signal transduction cascade through their ability to ubiquitinate tyrosine kinases including Src family kinases such as Fyn and Lck; Syk family kinases such as ZAP-70, Syk, PKC- $\theta$ , phospholipase C- $\gamma$ , and p85; and the regulatory subunit of PI3K (4, 5, 9–15).

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

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

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<sup>2</sup> The abbreviations used are: TCR, T cell receptor; IS, immunological synapse(s); OVA, ovalbumin; Ab, antibody; Ub, ubiquitin.

## GRAIL Regulates Cytoskeletal Reorganization

plays a major role in modulating autoimmunity in Fas<sup>lpr</sup> mice (30).

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

### EXPERIMENTAL PROCEDURES

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

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

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

**Constructs**—GRAIL, Arp2/3-5, coronin 1A, RhoGDIα, RhoGDIβ, Lasp1, and RGS10 cDNAs from DO11.10 T cells in which anergy had been induced by ionomycin were amplified with following the specific PCR primers: GRAIL, 5'-CAGTGAAATTCATGGGGCCGCGCCCGGGATC-3' and 5'-CAGTCTCGAGAGATTTAATCTCCCGAACAGCAGC-3'; Arp2/3-5, 5'-CATGGAATTCCTCCGGGATGTCGAAGAACACGGTGTC-3' and 5'-GATCGCGGCCGCCACGGTTTTCTT-

GCAGTCA-3'; coronin1A, 5'-GATCGCGGCCCGCTACTTGGCCTGAACAGTCTCCTC-3' and 5'-CAGTCTCGAGCTTGGCCTGAACAGTCTCCTC-3'; RhoGDIα, 5'-CATGGAATTCGTAAGCATGGCAGAACAGGAACCCAC-3' and 5'-GATCGCGGCCGCGTCTCCACTCCTTTTTGA-3'; RhoGDIβ, 5'-CATGGGATCCATCAAGATGACGGAGAAGGATGCAACA-3' and 5'-GATCGCGGCCGTTCTGTCCAATCCTTCTTAA-3'; and RGS10, 5'-CAGTGGATCCATGTTACCCGCGCCGTG-3' and 5'-CAGTCTCGAGTGTGTTGTAATTCTGGAGGCTCG-3'. SOD1 cDNA from brain was amplified with the following PCR primers: 5'-CAGTGAATTCATGGCGATGAAAGCGGTGTGC-3' and 5'-CAGTCTCGAGCTGCGCAATCCCAATCACTCC-3'. PCR products were cloned into a pcDNA4 V5/His vector or pcDNA4 Myc/His vector. The H297N and H300N mutations in the RING domain of murine GRAIL were generated using a PCR site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Deletion of the RING domain in murine GRAIL was generated using the following PCR primers: for the 5'-PCR product, CAGTGAATTCATGGGGCCGCGCCCGGGATC and CAGTTTCGAATCTCCATCAGG-GCCAATTTTC; and for the 3'-PCR product, CAGTTTCGAA-GTGTGACATTCTCAAAGCT and CAGTCTCGAGAGATTTAATCTCCCGAACAGCAGC. After these reactions, the DNAs were digested with BamHI and HpaI, and the fragments, which were WT-GRAIL-V5/His, H2N2-GRAIL-V5/His, ΔRF-GRAIL-V5/His, Arp2/3-5-Myc/His, coronin 1A-Myc/His, RhoGDIα-Myc/His or RhoGDIβ-Myc/His, were subcloned into a pMIG vector. After pcDNA4 WT-GRAIL-V5/His was digested with NheI and XhoI, the fragment was subcloned into pAcGFP N1 vector.

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

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

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

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

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

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

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

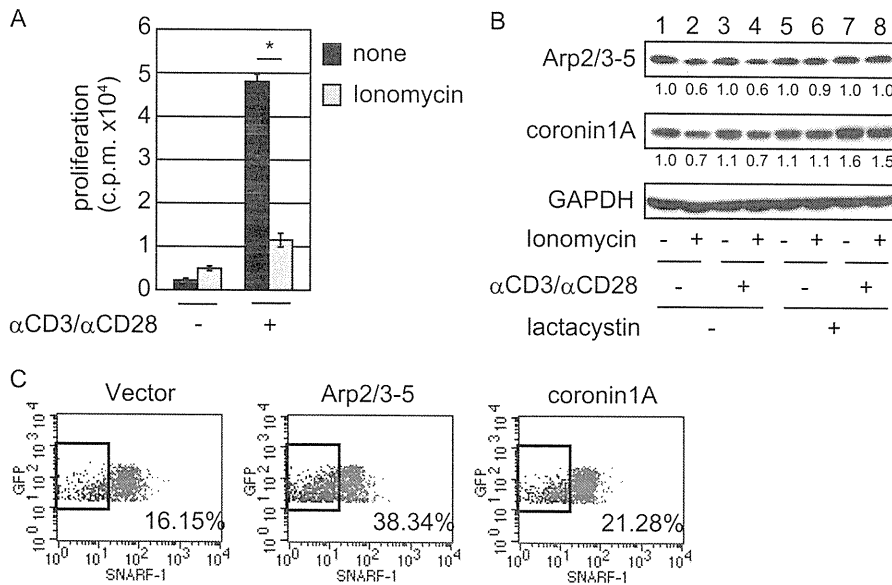
## RESULTS

**Reduced Expression of Arp2/3-5 and Coronin 1A**—E3 ubiquitin ligases including GRAIL are up-regulated in anergized T cells and play an important role in the induction of anergy (4, 8). To determine which proteins serve as substrates for GRAIL, we used two-dimensional difference gel electrophoresis to analyze proteins that were down-regulated in T cells in which anergy had been induced by ionomycin. Down-regulated proteins were identified by MALDI-TOF-MS and the nonredundant NCBI (NCBI nr) database using MASCOT software (supplemental Table S1). Proteins related to cytoskeletal reorganization were the most frequently down-regulated proteins in anergic T cells. We decided to focus on actin-related proteins Arp2/

3-5 and coronin 1A. We first confirmed that the expression levels of these proteins were reduced in T cells in ionomycin-induced anergy. We stimulated splenocytes of DO11.10 mice with OVA protein for 3 days and then rested them for 7 days. Anergy was induced by the addition of ionomycin for 18 h and the proliferative response upon the addition of anti-CD3 and anti-CD28 Abs detected by the incorporation of [<sup>3</sup>H]thymidine. The proliferative response was significantly suppressed in ionomycin-treated cells, confirming that anergy was properly induced (Fig. 1A). In these anergized cells, the protein expression of Arp2/3-5 and coronin 1A was reduced (Fig. 1B, lanes 2 and 4). To address the functional involvement of Arp2/3-5 and coronin 1A in T cell anergy, we examined whether overexpression of these proteins in DO11.10 CD4<sup>+</sup> T cells enhanced their proliferative response upon stimulation. DO11.10 CD4<sup>+</sup> T cells were transfected with Arp2/3-5 or coronin 1A. To analyze proliferation of transfected T cells by flow cytometry, the cells were treated with ionomycin and labeled with SNARF-1, which can monitor proliferating cells through dye dilution in a similar fashion to CFSE dilution assay. The number of proliferating cells upon stimulation (GFP<sup>+</sup> SNARF-1<sup>-</sup> cells) was increased in Arp2/3-5 or coronin 1A-overexpressing cells compared with that of control cells (Fig. 1C). We also analyzed whether an anergy-like state was displayed by knockdown of Arp2/3-5 or coronin 1A. The percentage of proliferation increase upon the restimulation with anti-CD3/anti-CD28 was decreased in Arp2/3-5 shRNA-expressing T cells (8%) and in coronin 1A shRNA-expressing T cells (3%) compared with that in control shRNA-expressing cells (13%). These results indicate that the expression of Arp2/3-5 and coronin 1A is correlated with T cell responses and is reduced in anergic T cells.

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

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**FIGURE 1. Arp2/3-5 and coronin 1A are down-regulated in T cells energized by ionomycin.** *A* and *B*, splenocytes derived from DO11.10 mice were stimulated with OVA for 3 days and rested for 7–10 days. The rested T cells were then treated with or without ionomycin for 18 h and restimulated with plate-bound anti-CD3 and soluble anti-CD28. *A*, proliferation was assessed by [<sup>3</sup>H]thymidine uptake for 48 h. The mean c.p.m. of triplicate wells  $\pm$  S.E. is shown ( $n = 9$ ). \*,  $p = 0.0000033$  versus control. *B*, cells were lysed and analyzed by immunoblotting after 1-hour activation with plate-bound anti-CD3 and soluble anti-CD28. Each protein level analyzed by ImageJ software was normalized to the corresponding GAPDH level and is expressed as relative quantity to that of untreated control. *C*, DO11.10 CD4<sup>+</sup> T cells were transfected with vector control (GFP alone), Arp2/3-5, or coronin 1A. Forty-eight hours later, the transfected cells were treated with ionomycin for 18 h and were labeled with SNARF-1. The cells were restimulated with plate-bound anti-CD3 and soluble anti-CD28. Forty-eight hours later, proliferation was analyzed by FACS.

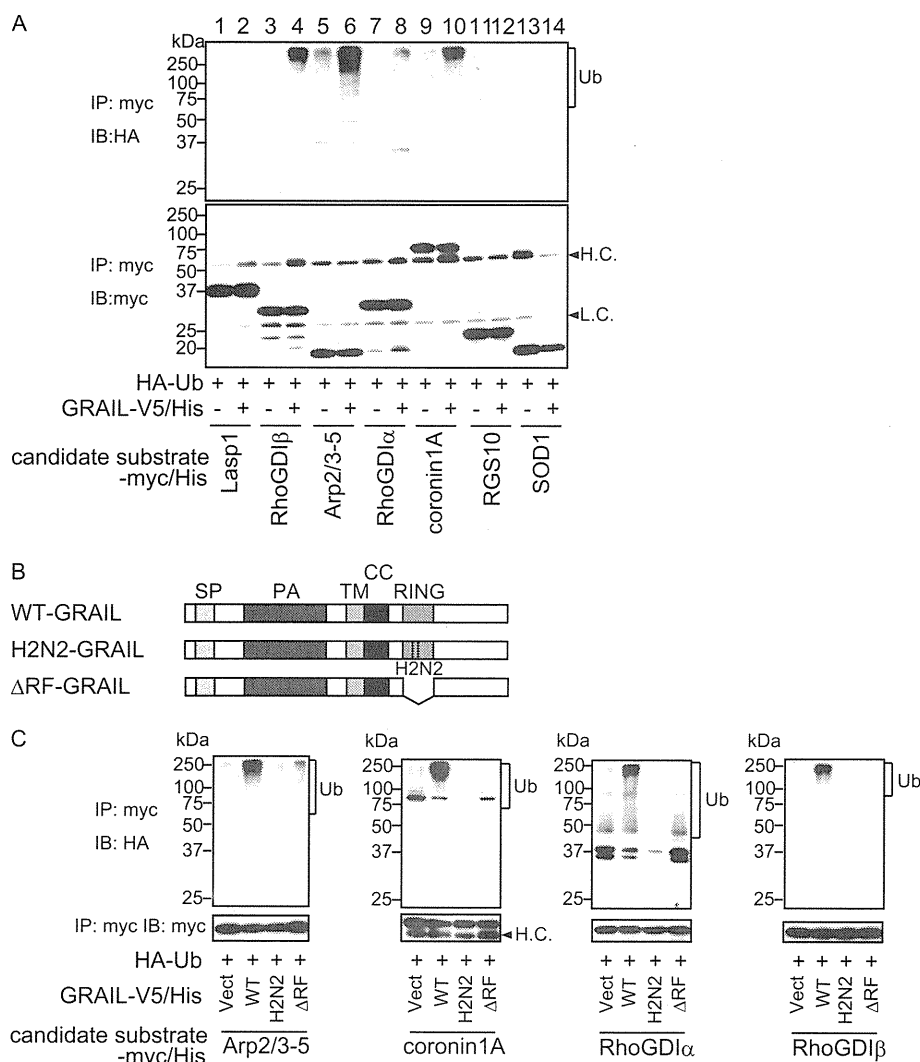
dence suggests that Cbl-b, which is E3 ligase as well as GRAIL, is important for induction of T cell anergy. We also analyzed whether Arp2/3-5 and coronin 1A are substrates of Cbl-b. However, Arp2/3-5 and coronin 1A are not ubiquitinated by Cbl-b (supplemental Fig. S1). These data indicate that GRAIL but not Cbl-b E3 ligase selectively ubiquitinates Arp2/3-5 and coronin 1A.

**GRAIL Co-localizes with Arp2/3-5 and Coronin 1A**—To address the interaction of Arp2/3-5 and coronin 1A with GRAIL, we examined the co-localization of these proteins. We transiently expressed GFP-tagged GRAIL together with HA-tagged ubiquitin and DsRed-tagged Arp2/3-5, coronin 1A, or RhoGDI $\alpha/\beta$ . After treatment with lactacystin, the localization of GRAIL and its substrates was analyzed by confocal microscopy. Indeed, Arp2/3-5 (Fig. 3*A*), coronin 1A (Fig. 3*B*), and RhoGDI $\alpha$  and  $\beta$  (Fig. 3, *C* and *D*) all co-localized with GRAIL. The substrates were localized together with GRAIL in contrast to the diffuse localization of GFP and substrate proteins in the cells transfected with GFP control vector and substrate proteins, indicating the co-localization of GRAIL and Arp2/3-5 or coronin 1A. These findings suggest that Arp2/3-5 and coronin 1A interact with GRAIL.

**GRAIL Ubiquitinates Arp2/3-5 and Coronin 1A via Lys-63 and Lys-48**—GRAIL has been reported to form polyubiquitin chains through lysine 63, resulting in proteolysis-independent functional modulation of Rho GDIs. However, when CD151 is the substrate, polyubiquitin chains are formed through lysine 48, which leads to protein degradation (18). We therefore assessed whether GRAIL ubiquitinates Arp2/3-5 and coronin 1A through Lys-63 and Lys-48. A similar polyubiquitinated ladder pattern of Arp2/3-5 was observed in the presence of WT Ub

or Ub containing a lysine to arginine substitution at residue 29 (K29R) (Fig. 4*A*, lanes 4 and 6). In contrast, Ub conjugation of Arp2/3-5 was barely detected in the presence of Ub containing a lysine to arginine substitution at residue 48 (K48R) or at residue 63 (K63R) (Fig. 4*A*, lanes 8 and 10). Similarly, Ub conjugation of coronin 1A was observed in the presence of WT or K29R Ub (Fig. 4*B*, lanes 4 and 6) but was much lower when K48R or K63R Ub was used (Fig. 4*B*, lanes 8 and 10). These data reveal that Arp2/3-5 and coronin 1A were modified by Lys-48 and Lys-63 mixed linkage ubiquitin chains. To address the effect of GRAIL on the protein levels of Arp2/3-5 and coronin 1A, we overexpressed GRAIL and its enzymatically inactive mutant, H2N2-GRAIL or  $\Delta$ RF-GRAIL, in DO11.10 CD4<sup>+</sup> T cells and determined Arp2/3-5 and coronin 1A expression by immunoblotting with specific Abs. Both Arp2/3-5 and coronin 1A were reduced when GRAIL, but not the enzymatically inactive forms of GRAIL, was overexpressed (Fig. 4*C*). These results indicate that GRAIL polyubiquitinates Arp2/3-5 and coronin 1A through Lys-48 and Lys-63 and eventually leads them to be degraded.

**Less Arp2/3-5 and Coronin 1A Localize at the IS in Anergy**—To investigate the role of Arp2/3-5 and coronin 1A in anergic T cells, we next examined the accumulation of F-actin, Arp2/3-5, and coronin 1A at the IS using confocal microscopy. As described previously, F-actin and Arp2/3-5 were recruited to the IS formed between DO11.10 CD4<sup>+</sup> T cells and OVA<sub>323–339</sub> peptide-pulsed A20 B cells (Fig. 5, *A* and *B*, top panels). In contrast, the accumulation of F-actin and the recruitment of Arp2/3-5 to the IS were reduced in ionomycin-treated DO11.10 CD4<sup>+</sup> T cells compared with those in control cells (Fig. 5*A*, bottom panel). Similarly, the recruitment of coronin 1A to the



**FIGURE 2. Arp2/3-5 and coronin 1A are ubiquitinated by GRAIL.** *A* and *C*, HEK293T cells were transiently transfected with the indicated constructs and were treated with lactacystin for 12 h before lysis. Ubiquitination of the indicated proteins was detected by immunoprecipitation (IP) with anti-Myc Ab, followed by anti-HA immunoblotting (IB). The membrane was stripped and reprobed with anti-Myc Ab. *B*, schematic structures of the WT-, H2N2-, and ΔRF-GRAIL proteins.

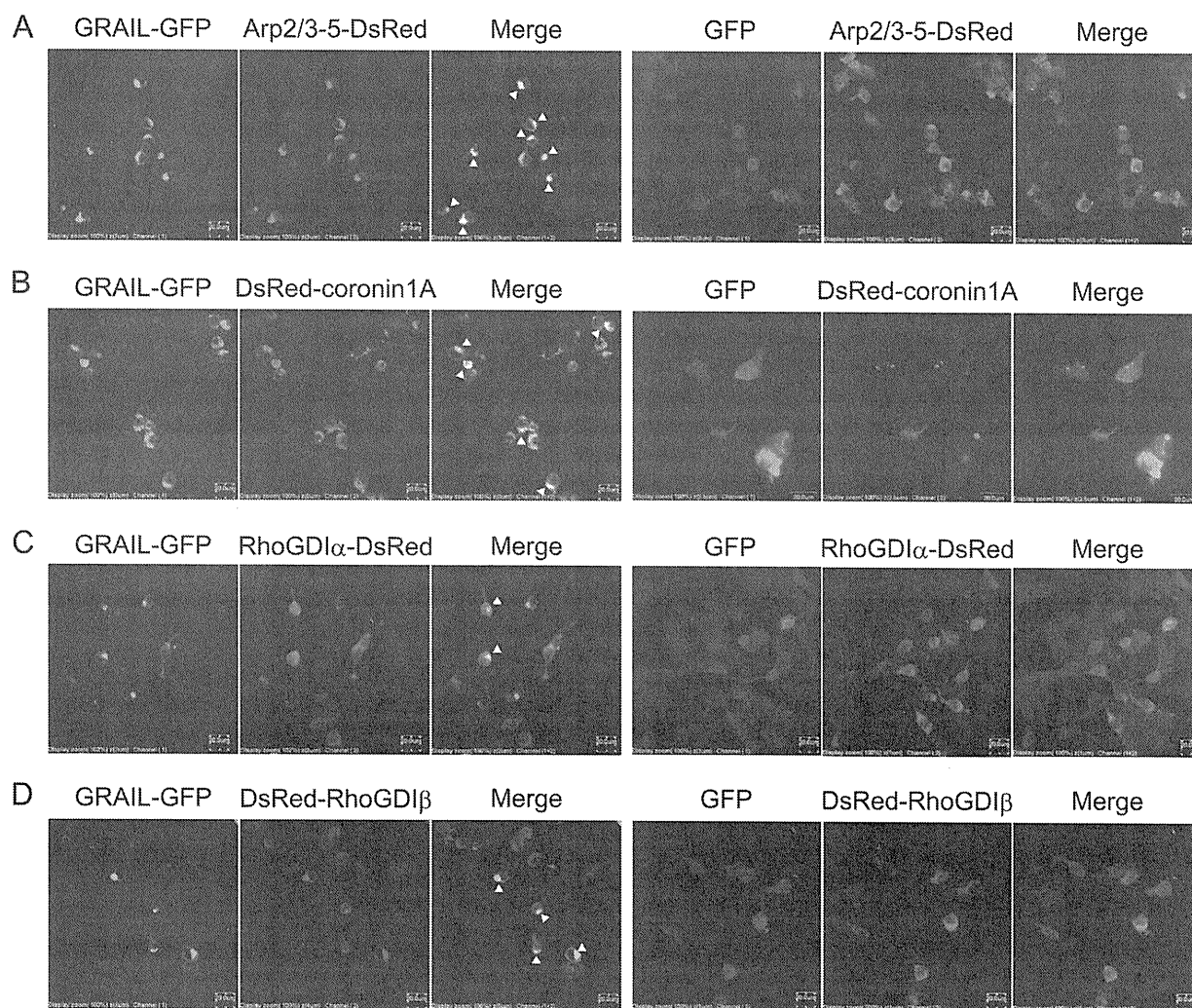
IS in ionomycin-treated DO11.10 CD4<sup>+</sup> T cells was reduced compared with that in nontreated DO11.10 CD4<sup>+</sup> T cells (Fig. 5*B*, bottom panel). These data demonstrate that the accumulation of Arp2/3-5 and coronin 1A together with F-actin at the IS is impaired in anergic T cells.

**GRAIL Inhibits Arp2/3 and Coronin 1A Accumulation at the IS**—To address the contribution of GRAIL to IS formation, we overexpressed GRAIL, ΔRF-GRAIL, or a control vector in DO11.10 CD4<sup>+</sup> T cells and analyzed the accumulation of Arp2/3-5, coronin 1A, and F-actin at the IS. First, the expression of Arp2/3-5 and coronin 1A was reduced in T cells (GFP-positive cells) in which GRAIL was overexpressed compared with expression levels in control cells (Fig. 6, *A* and *B*, compare top and middle panels). The accumulation of both Arp2/3-5 and coronin 1A together with F-actin was reduced in DO11.10 CD4<sup>+</sup> T cells overexpressing GRAIL compared with that in control vector-transfected T cells (Fig. 6, *A* and *B*, compare top and middle panels). On the other hand, the accumulation of Arp2/3-5, coronin 1A, and F-actin at the IS in DO11.10 CD4<sup>+</sup> T

cells overexpressing ΔRF-GRAIL was similar to that in controls (Fig. 6, *A* and *B*, bottom panels). We also examined whether the formation of IS occurred in ionomycin-treated T cells in which GRAIL was down-regulated by GRAIL shRNA-encoding retroviral infection. Coincident with the results for GRAIL-overexpressing experiments, both Arp2/3-5 and coronin 1A together with F-actin fully accumulated at the IS in ionomycin-treated GRAIL knockdown DO11.10 CD4<sup>+</sup> T cells compared with that in ionomycin-treated control T cells (anergic T cells) (supplemental Fig. S2). These results indicated that GRAIL regulates the recruitment of Arp2/3-5 and coronin 1A into the IS and the subsequent accumulation of F-actin at the site of the IS.

**GRAIL Inhibits Lamellipodium Formation**—Because Arp2/3 has been reported to be essential for the formation of lamellipodia at the IS, we next examined the effect of GRAIL on lamellipodium formation. Because the spreading of T cells on anti-TCR-coated coverslips requires the formation of stable actin structures and the generation of lamellipodia, we first analyzed whether T cells could spread onto anti-CD3-coated coverslips

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**FIGURE 3. GRAIL co-localizes with Arp2/3-5 and coronin 1A.** HEK293T cells were transiently transfected with constructs expressing GFP-tagged GRAIL, DsRed-tagged substrates (Arp2/3-5, *A*; coronin1A, *B*; RhoGDI $\alpha$ , *C*, and RhoGDI $\beta$ , *D*), and HA-ubiquitin and were treated with lactacystin for 12 h before being fixed. Co-localization with GFP-GRAIL was analyzed by confocal microscopy.

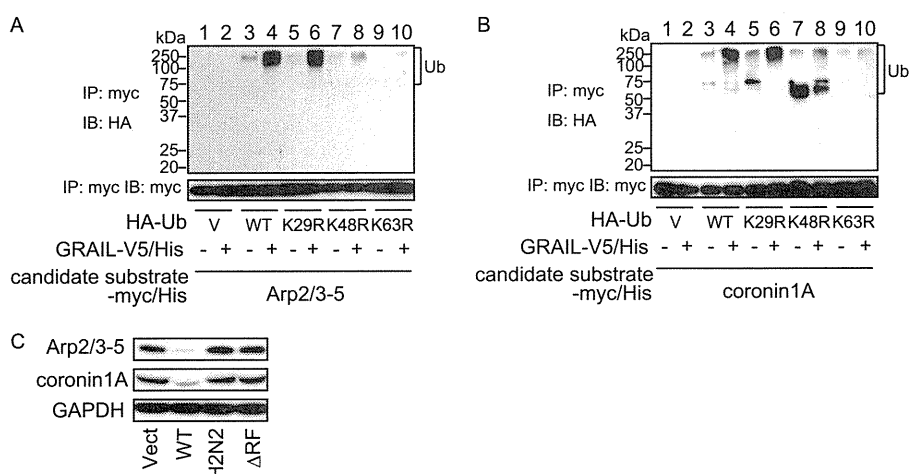
under anergic conditions. Control DO11.10 CD4<sup>+</sup> T cells spread onto anti-TCR-coated coverslips and formed round lamellipodial interfaces containing F-actin-rich structures (Fig. 7A). In contrast, DO11.10 CD4<sup>+</sup> T cells in which anergy had been induced by ionomycin barely formed lamellipodia (Fig. 7A, *bottom panels*). We next analyzed the lamellipodium formation in CD4<sup>+</sup> T cells overexpressing GRAIL. Lamellipodia were not efficiently formed on anti-CD3-coated coverslips when GRAIL was overexpressed in DO11.10 CD4<sup>+</sup> T cells (Fig. 7B, *middle panels*). In contrast, lamellipodia were efficiently formed at the IS when a catalytically inactive mutant GRAIL ( $\Delta$ RF) was overexpressed in DO11.10 CD4<sup>+</sup> T cells (Fig. 7B, *bottom panels*). These data demonstrate that GRAIL inhibits lamellipodium formation at the IS.

### DISCUSSION

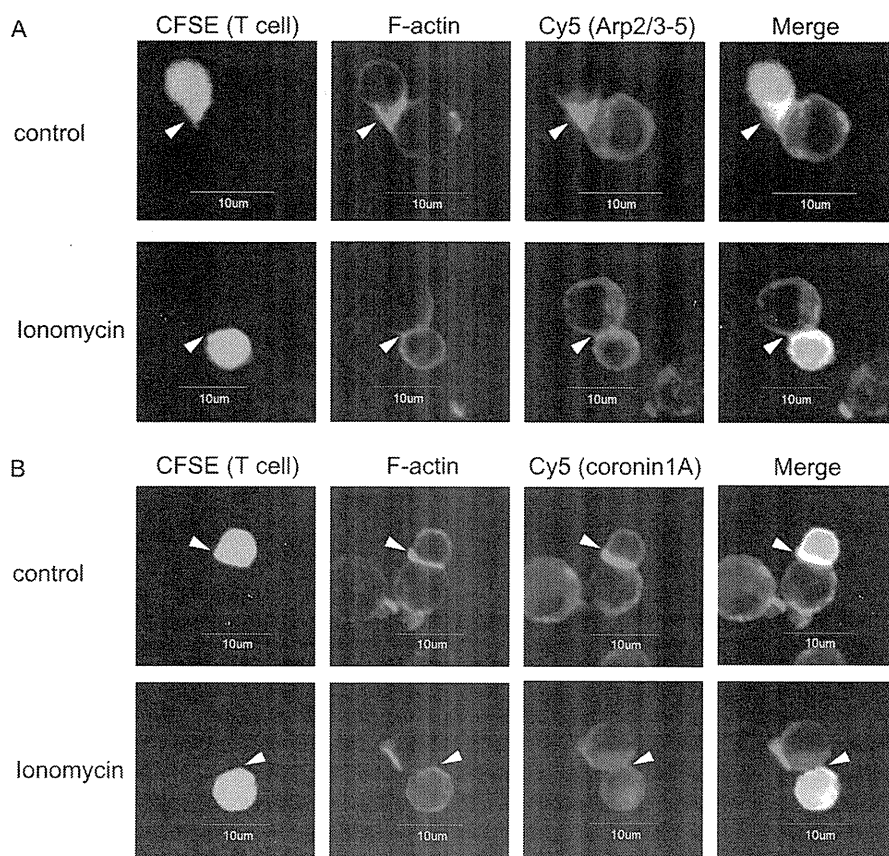
In this study, we demonstrate that Arp2/3-5 and coronin 1A are down-regulated in anergic T cells as well as in T cells that overexpress GRAIL. Arp2/3-5 and coronin 1A co-localize with

GRAIL and are ubiquitinated by GRAIL but not by Cbl-b via Lys-48 and Lys-63 linkage. Furthermore, the accumulation of Arp2/3-5 and coronin 1A together with F-actin is reduced at the IS in anergic T cells or in T cells that overexpress GRAIL. Coincident with the results for GRAIL-overexpressing experiments, IS formation in ionomycin-treated anergic T cells occurred by knockdown of GRAIL. Finally, we showed that overexpression of GRAIL suppresses lamellipodium formation at the IS.

CD40 ligand, CD151, CD83, and RhoGDI have been reported to be candidate substrates of GRAIL; however, the mechanism of GRAIL-mediated anergy induction is not yet fully understood (18–21). In fact, the expression of CD40 ligand was not up-regulated, and the down-regulation of CD3 was impaired in GRAIL-deficient mice. Because GRAIL is the only membrane protein among E3 ligases up-regulated in anergic T cells, it is reasonable that membrane proteins such as CD151 or CD83 are regulated by GRAIL. In this study, we confirmed that cytosolic proteins such as RhoGDIs serve as substrates for GRAIL. Fur-



**FIGURE 4. Arp2/3-5 and coronin 1A are polyubiquitinated through Lys-48 and/or Lys-63 ubiquitin linkages and are down-regulated by catalytically active GRAIL.** *A* and *B*, HEK293T cells were transiently transfected with the indicated vectors and were treated with lactacystin for 12 h before lysis. Arp2/3-5 (*A*) and coronin 1A (*B*) were immunoprecipitated (IP) with anti-Myc Ab followed by immunoblotting (IB) with anti-HA Ab. The membrane was stripped and reprobed with anti-Myc Ab. *C*, CD4<sup>+</sup> T cells were transfected with vector control (GFP alone) or WT-, H2N2-, or ΔRF-GRAIL expression constructs. Forty-eight hours later, the transfected cells (GFP<sup>+</sup> cells) were sorted using a FACS Aria cell sorter. Sorted cells were rested for 2 days and were subjected to immunoblot analysis with anti-coronin 1A or Arp2/3-5 Ab.

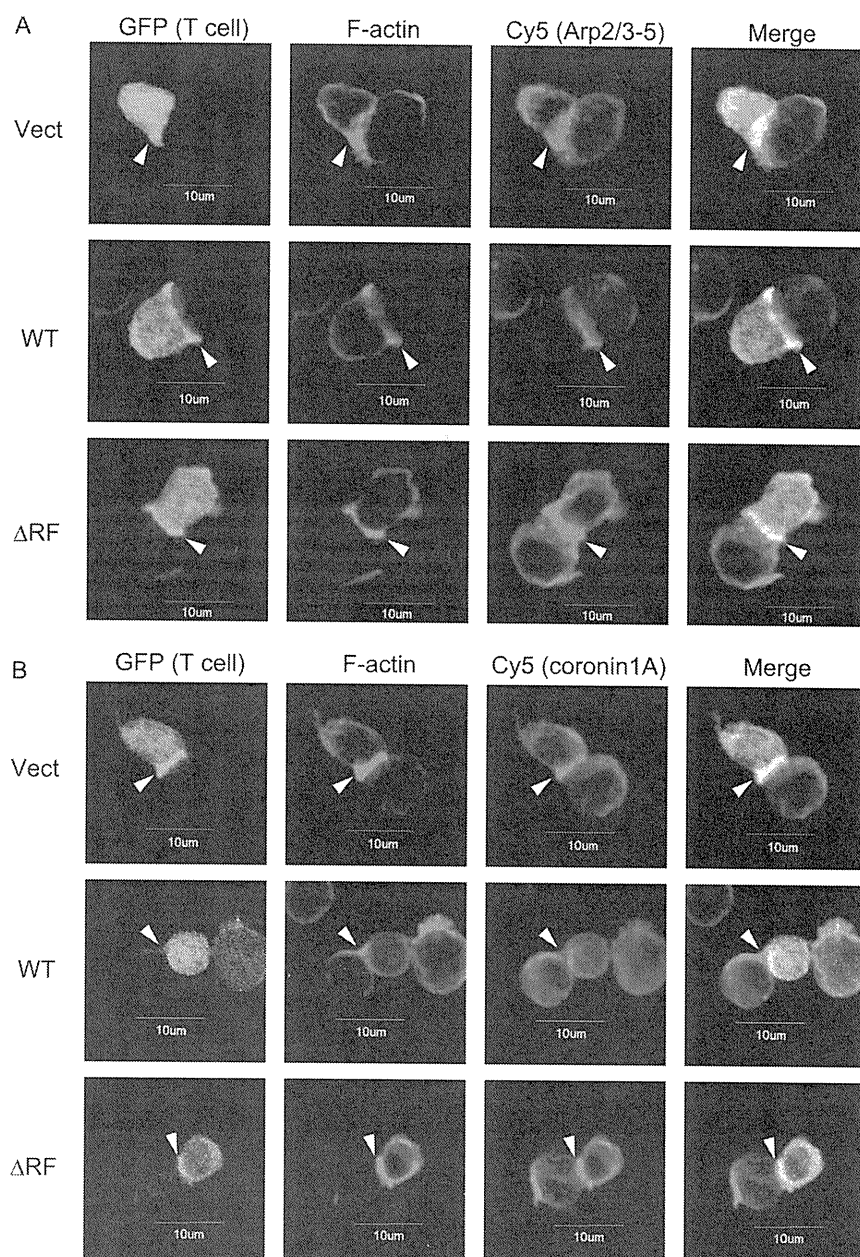


**FIGURE 5. The accumulation of Arp2/3-5, coronin 1A, and F-actin at the IS is reduced in anergic T cells.** *A* and *B*, OVA-stimulated DO11.10 splenocytes were rested for 7–10 days. Rested T cells were stained with CFSE, treated with or without ionomycin for 18 h, incubated with OVA<sub>323–339</sub>-pulsed A20 cells, and co-stained with rhodamine-phalloidin (red) to visualize F-actin and either anti-Arp2/3-5 Ab (*A*) or anti-coronin 1A Ab (purple) (*B*). The arrowheads indicate IS.

thermore, we identified Arp2/3-5 and coronin 1A as novel substrates for GRAIL. Interestingly, these proteins as well as RhoG-DIs are reportedly involved in the regulation of cytoskeletal organization. Although ubiquitination of target proteins was

almost completely lost when either K63R or K48R mutant ubiquitin was used, it remains unclear whether Arp2/3-5 and coronin 1A are ubiquitinated via Lys-48, Lys-63, or both sites. To address this issue, characterization of ubiquitin chain using

## GRAIL Regulates Cytoskeletal Reorganization



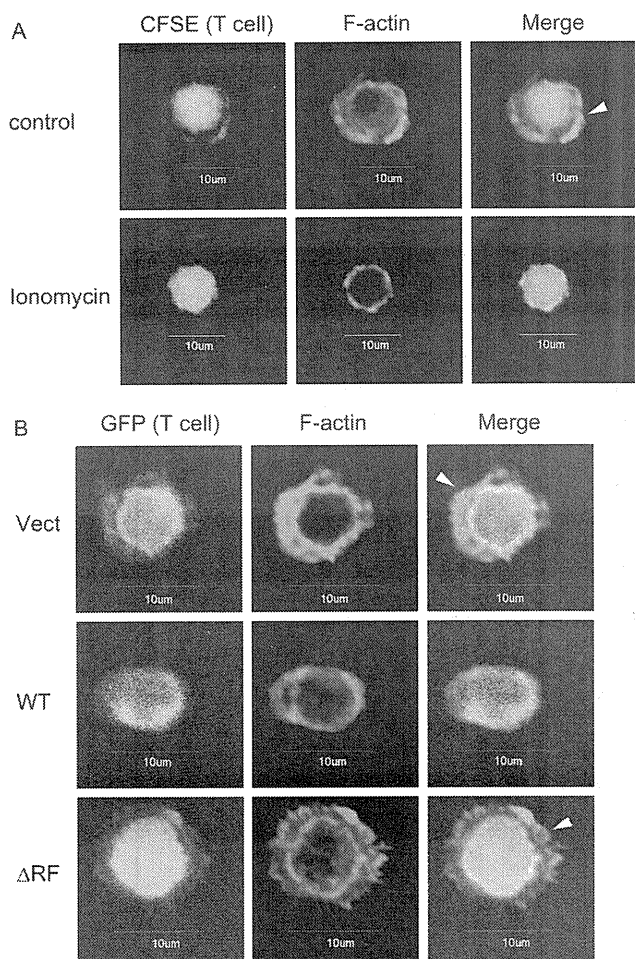
**FIGURE 6. GRAIL inhibits the accumulation of Arp2/3-5, coronin 1A, and F-actin at the IS.** *A* and *B*, DO11.10 CD4<sup>+</sup> T cells were transfected with vector control (GFP alone) or WT- or ΔRF-GRAIL expression constructs (green). Each population was incubated with OVA<sub>323–339</sub>-pulsed A20 cells and co-stained with rhodamine-phalloidin (red) and either anti-Arp2/3-5 (*A*) or anti-coronin 1A (purple) (*B*). The arrowheads indicate IS.

MALDI-TOF-MS or mutants in which Lys-48 or Lys-63 is the only lysine residue that can mediate the ubiquitin chain formation will be needed for future studies

The immunological synapse is important in sustained signaling and delivery of a subset of effector cytokines by CD4<sup>+</sup> T cells (25, 29, 31, 32). Although the precise contribution of actin cytoskeletal remodeling to T cell signaling and biologic function is not completely understood, both anergic T cells and T cells overexpressing GRAIL have been reported to form unstable immunologic synapses (4, 38). Actin nucleation in T cells is induced by the WAVE2 complex (33) and the actin-nucleation-promoting factor WASPs, which are required to promote and stabilize interactions between T cells and APC *in vitro* and TCR

clustering on artificial surfaces. WASPs bind to actin monomers, whereas the acidic stretch associates with the Arp2/3-5 complex (23, 34), a seven-subunit complex that has intrinsic actin-nucleating activity and is essential for polarization of F-actin at the IS (25, 35). In addition, co-localization of WASPs and the Arp2/3-5 complex at the interface between anti-CD3-coated beads and Jurkat T cells suggests that these cytoskeletal components are essential for the dynamics of the actin cytoskeleton and for T cell function (24). Arp2/3-5 is essential for the formation of a stable synapse by creating lamellipodia (25). Consistent with these findings, overexpression of GRAIL reduced the protein expression of Arp2/3-5 and impaired lamellipodium formation. These results suggest that proteins





**FIGURE 7. GRAIL inhibits lamellipodium formation during TCR stimulation.** A, OVA-stimulated DO11.10 splenocytes were rested for 7–10 days and stained with CFSE. The cells were treated with or without ionomycin for 18 h. The cells were stimulated with plate-bound anti-CD3 mAb and stained with rhodamine-phalloidin (red) to visualize F-actin. B, DO11.10 CD4<sup>+</sup> T cells were transfected with a control vector (GFP alone) or WT- or  $\Delta$ RF-GRAIL expression vectors. The cells were stimulated with coated anti-CD3 mAb and stained with rhodamine-phalloidin (red). The arrowheads indicate lamellipodium formation.

related to cytoskeletal reorganization at the IS are cytosolic targets for GRAIL.

An earlier study of coronin 1A knock-out mice reported that coronin 1A has an Arp2/3-5-dependent inhibitory effect on F-actin formation and concluded that coronin 1A is indispensable for TCR signaling (27, 29). In the present study, overexpression of coronin 1A restored the proliferative response. These findings suggest that coronin 1A participates in modulating T cell signaling and thereby contributes to the maintenance of anergy. In anergic T cells and in T cells overexpressing GRAIL, F-actin accumulation at the IS was decreased, although the expression of coronin 1A was reduced in contrast to previous studies. This may be because GRAIL regulates not only coronin 1A but also the Arp2/3-5 complex as well as RhoGDI, which are important in the regulation of the accumulation of F-actin.

Anergic T cells have been reported to exhibit initial interaction, but implementation of T cell anergy results in reduced

binding of LFA-1 to its ligand ICAM-1 (4). This process is mediated through degradation of PKC- $\theta$  and phospholipase C- $\gamma$  by Cbl-b. A recent report demonstrated that overexpression of GRAIL impairs LFA-1 polarization at the IS (37). Stimulation through the TCR was shown to result in WAVE2-Arp2/3-5-dependent F-actin nucleation and the formation of a complex containing WAVE2, Arp2/3-5, vinculin, and talin (33). Moreover, TCR stimulation induces integrin clustering through the recruitment of vinculin and talin (33). Therefore, our study might link the unstable immunological synapse formation and impaired LFA-1 polarization at the IS in anergic T cells. Thus, whereas Cbl-b leads to unstable immunological synapse through degradation of tyrosine kinase, GRAIL leads to the phenotype of synapse disorganization via degradation of proteins involved in the actin cytoskeletal organization. In summary, we provide evidence that GRAIL regulates cytoskeletal reorganization to keep cells unresponsive to further antigen stimulation through the ubiquitination and down-regulation of the Arp2/3-5 complex and coronin 1A.

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## Non-phosphorylated FTY720 Induces Apoptosis of Human Microglia by Activating SREBP2

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**Abstract** A synthetic analog of sphingosine named FTY720 (Fingolimod), phosphorylated by sphingosine kinase-2, interacts with sphingosine-1-phosphate (S1P) receptors expressed on various cells. FTY720 suppresses the disease activity of multiple sclerosis (MS) chiefly by inhibiting S1P-dependent egress of autoreactive T lymphocytes from secondary lymphoid organs, and possibly by exerting anti-inflammatory and neuroprotective effects directly on brain cells. However, at present, biological effects of FTY720 on human microglia are largely unknown. We studied FTY720-mediated apoptosis of a human microglia cell line HMO6. The exposure of HMO6 cells to non-phosphorylated FTY720 (FTY720-non-P) induced apoptosis in a dose-dependent manner with IC<sub>50</sub> of  $10.6 \pm 2.0 \mu\text{M}$ , accompanied by the cleavage of

caspase-7 and caspase-3 but not of caspase-9. The apoptosis was inhibited by Z-DQMD-FMK, a caspase-3 inhibitor, but not by Pertussis toxin, a Gi protein inhibitor, suramin, a S1P3/S1P5 inhibitor, or W123, a S1P1 competitive antagonist, although HMO6 expressed S1P1, S1P2, and S1P3. Furthermore, both phosphorylated FTY720 (FTY720-P) and SEW2871, S1P1 selective agonists, did not induce apoptosis of HMO6. Genome-wide gene expression profiling and molecular network analysis indicated activation of transcriptional regulation by sterol regulatory element-binding protein (SREBP) in FTY720-non-P-treated HMO6 cells. Western blot verified activation of SREBP2 in these cells, and apoptosis was enhanced by pretreatment with simvastatin, an activator of SREBP2, and by overexpression of the N-terminal fragment of SREBP2. These observations suggest that FTY720-non-P-induced apoptosis of HMO6 human microglia is independent of S1P receptor binding, and positively regulated by the SREBP2-dependent proapoptotic signaling pathway.

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### Abbreviations

CNS	Central nervous system
DAVID	Database for Annotation, Visualization, and Integrated Discovery
EDG	Endothelial differentiation gene
FTY720-non-P	Non-phosphorylated form of FTY720
FTY720-P	Phosphorylated form of FTY720
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein-coupled receptor
INSIG1	Insulin-induced gene 1
LDLR	Low density lipoprotein receptor

MS	Multiple sclerosis
OPC	Oligodendroglial progenitor cell
S1P	Sphingosine-1-phosphate
SPHK2	Sphingosine kinase-2
SREBP	Sterol regulatory element-binding protein
PARP	Poly-ADP-ribose-polymerase
PTX	Pertussis toxin

## Introduction

FTY720 (Fingolimod) is a synthetic analog of sphingosine generated by chemical modification of myriocin, a natural product of the fungus *Isaria sinclairii*. FTY720, phosphorylated by endogenous sphingosine kinase-2 (SPHK2), is converted into the biologically active form FTY720-P that binds to sphingosine-1-phosphate (S1P) receptors expressed on various cells (Brinkmann et al. 2010). S1P receptors belong to the endothelial differentiation gene (EDG) receptor family of G-protein-coupled receptors (GPCRs). FTY720-P interacts with S1P1, S1P3, S1P4, and S1P5 but not with S1P2. S1P1, S1P2, and S1P3 are distributed widely in the immune system, cardiovascular system, and the central nervous system (CNS), and S1P4 expression is more restricted to the lung, spleen, and thymus, while S1P5 is located chiefly on the skin, spleen, and brain. FTY720-P not only serves as an agonist for S1P receptors, but also acts as a functional antagonist for S1P1 by downregulating the receptor expression via internalization, ubiquitination, and proteasomal degradation (Mullershausen et al. 2009). The latter induces unresponsiveness to endogenous S1P.

Recent clinical trials indicate that FTY720 has promising therapeutic effects on multiple sclerosis (MS), a human demyelinating disease affecting exclusively the CNS white matter (Brinkmann et al. 2010). Oral administration of FTY720 reduces the number of gadolinium-enhanced lesions on MRI and decreased annual relapse rate in the patients with relapsing-remitting MS (Kappos et al. 2006). Consequently, US Food and Drug Administration (FDA) approved FTY720 as the first oral medication for MS in September 2010. FTY720-mediated immunomodulatory effects on the disease activity of MS are chiefly attributable to inhibition of S1P-dependent egress of autoreactive T lymphocytes from secondary lymphoid organs (Brinkmann et al. 2010).

Increasing evidence indicates that FTY720, capable of passing the blood–brain barrier due to its lipophilicity, exerts anti-inflammatory and neuroprotective effects within the CNS by interacting with a battery of S1P receptors expressed on neural cells (Dev et al. 2008). Reactive

astrocytes in active MS lesions show a robust increase in S1P1 and S1P3 expression, where FTY720-P inhibits production of proinflammatory cytokines from astrocytes (Van Doorn et al. 2010). FTY720-P persistently downregulates S1P1 expression on astrocytes, and thereby attenuates the disease activity of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Choi et al. 2011). FTY720-P induces rapid phosphorylation of ERK1/2 and activates the PI3-kinase/Akt pathway in rat oligodendrocyte progenitor cells (OPCs), and subsequently protects OPCs from apoptosis caused by proinflammatory mediators (Coelho et al. 2007). FTY720-P promotes process extension of human OPCs and enhances their survival (Miron et al. 2008).

Microglia, acting as antigen-presenting cells and proinflammatory effector cells in the CNS, play a central role in development of demyelinating lesions in MS (Jack et al. 2005). Therefore, it is possible that FTY720 acts directly on microglia at the site of inflammation in MS brains. S1P1-expressing cells positive for CD68, a marker of microglia/macrophages, are accumulated in MS lesions (Van Doorn et al. 2010). Rat microglial cells express mainly S1P1 and S1P3 (Dev et al. 2008). In mouse organotypic cerebellar cultures affected with lysolecithin-induced demyelination, FTY720-P induces proliferation of microglia (Miron et al. 2010), while FTY720 reduces the accumulation of reactive microglia/macrophages in the lesions of traumatic brain injury (Zhang et al. 2007). FTY720 reduces the lesion size of cerebral infarct in mice with middle cerebral artery (MCA) occlusion and improves neurological deficits, accompanied by a decrease in the number of activated microglia/macrophages and apoptotic neurons (Wei et al. 2011). FTY720-P does not affect the global cytokine production by cultured human microglia (Durafourt et al. 2011). However, at present, immunomodulatory effects of FTY720 on human microglia remain largely unknown. The aim of the present study is to investigate biological effects of FTY720 on a human microglial cell line HMO6.

## Methods

### Human Microglia Cell Line HMO6

The HMO6 cell line was established by immortalizing cultured microglia isolated from human embryonic telencephalon tissues with a retroviral vector PASK1.2 encoding v-myc oncogene (Nagai et al. 2001). HMO6 cells express the markers of the microglia/macrophage lineage cells, including CD11b, CD68, CD86, HLA-ABC, HLA-DR, and ricinus communis agglutinin lectin-1 (RCA), serving as a model of human microglia both in