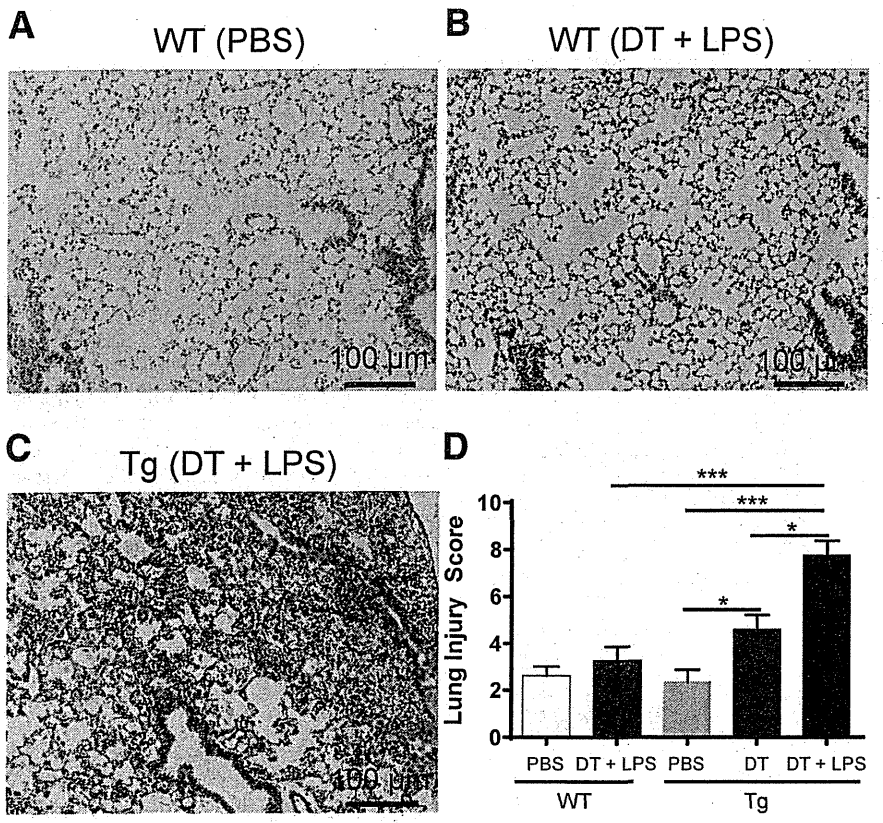

Figure 4 Lung sections stained with H&E. **A:** Control WT mice. **B:** DT-treated WT mice who received LPS. **C:** DT-treated CD206-DT receptor Tg mice who received LPS. **D:** Semiquantitative analysis of lung tissues by lung injury score. n = 5 to 7 (**D**). $^{*}P < 0.05$, $^{***}P < 0.001$.

receptor is highly regulated and correlates with the functional state of macrophages. Thus, fully mature activated M2 macrophages express CD206 as a phenotypic hallmark.[39] Therefore, CD206 can be widely used to identify the M2 phenotype.[3] The use of M2 macrophage markers Ym1 and arginase-1 identified pulmonary CD206-positive cells as M2 macrophages. Furthermore, this was confirmed by our immunofluorescence labeling study showing that CD206-positive cells in BALF were labeled with arginase-1. Herein, DT administration on CD206-DT receptor Tg mice led to conditional CD206-positive cell ablation, which implies that pulmonary macrophages, which are M2 polarized under homeostatic conditions, can be effectively depleted *in vivo*.

Many M2 macrophages were found in the BALF of C57BL/6 mice under normal conditions. Lung sections from normal C57BL/6 mice showed that M2 macrophages were also detected in the alveoli. DT-mediated M2 macrophage depletion in CD206-DT receptor Tg mice demonstrated that mRNA levels of proinflammatory cytokines, IL-1β, TNF-α, MCP-1, and IL-6, were up-regulated in lungs. We interpret these observations to indicate that M2 macrophages lead to a tonic inhibition of pulmonary production of proinflammatory cytokines. Blood levels of IL-1β, MCP-1, and IL-6 were apparently increased when abolition of M2 macrophages was made by administration of DT to CD206-DT receptor Tg mice. This suggests that M2 macrophage-mediated tonic inhibition of the genesis of proinflammatory cytokines under basal subinflammatory conditions may be found systemically.

Administration of LPS at a low dose did not substantially alter expression of CD206 mRNA in mouse lungs, suggesting that M2 macrophages may be unchanged by a low dose of LPS. In contrast, a recent report has shown that low doses of LPS can effectively suppress expression of the M2 marker, arginase-1, when bone marrow–derived macrophages were treated with M2-skewing mediators.[40] This report suggests that M2 macrophages may be transformed into M1 macrophages after LPS stimulation to promote inflammation. However, our present study indicates that the presence of M2 macrophages is important in the regulation of the inflammatory state during endotoxemia. DT-mediated depletion of M2 macrophages in CD206-DT receptor Tg mice led to a discernible enhancement of the LPS-induced increases in pulmonary mRNA levels of proinflammatory cytokines. Furthermore, this manipulation significantly aggravated LPS-induced lung injury. These results strongly suggest that M2 macrophages serve as a protective phenotype and can lessen lung inflammation during endotoxemia. DT treatment accelerated the deleterious effect of LPS on blood proinflammatory cytokine levels in CD206-DT receptor Tg mice, implying that M2 macrophages appear essential for appropriate moderation of systemic endotoxemic inflammation.

M2 macrophages may produce some anti-inflammatory cytokines, as typified by IL-10, and maximize the IL-10

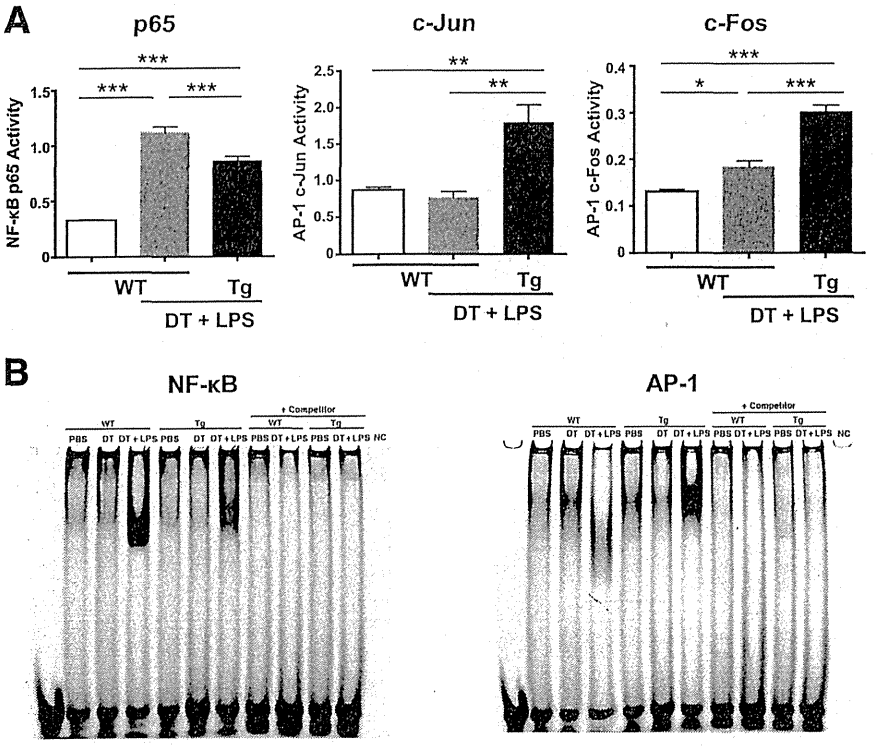

Figure 5 Activation of proinflammatory transcriptional factors in lungs of DT-treated WT and CD206-DT receptor Tg mice who were challenged with LPS. **A:** NF-κB p65, AP-1 c-Jun, and AP-1 c-Fos DNA binding activities assessed using TransAM NF-κB and TransAM AP-1 kits. **B:** Gel mobility shift assays for NF-κB and AP-1—binding activity. The induced NF-κB and AP-1 shift bands are indicated. Complete competition for binding of NF-κB and AP-1 is shown in the presence of excess unlabeled NF-κB and AP-1 oligodeoxynucleotides, respectively. Values are means ± SEM (**A**). $n = 5$ to 7 mice (**A**). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. NC, negative control.

signal to dampen the immune response. However, we observed that when LPS was challenged, the up-regulation of IL-10 mRNA expression was increased, rather than decreased, in CD206-depleted mice compared with WT. Thus, CD206-positive M2 macrophages are unlikely to be the principle source of production of IL-10 in lungs.

We found a notable increase in neutrophils in BALF cell contents in DT-treated CD206-DT receptor Tg mice. This suggests that the depletion of M2 macrophages altered inflammatory cell recruitment to the lung. This may account for the enhanced response to LPS in lungs of DT-treated CD206-DT receptor Tg mice.

In quiescent cells, NF-κB is maintained in inactive form by IκB.[41] LPS stimulates IκB-kinase that specifically phosphorylates IκB, resulting in IκB polyubiquitination and subsequent degradation, followed by liberation of NF-κB.[42] Many M1 genes have κB sites in their promoter region, including inducible nitric oxide synthase and cyclooxygenase 2.[43] Thus, in M1 macrophages, NF-κB orchestrates the expression of many proinflammatory genes in response to LPS. Herein, we showed that M2 macrophage depletion resulted in a significant reduction in LPS-induced NF-κB activity, suggesting that M2 phenotype-dependent counteraction of inflammatory insult cannot be attributed to the inhibition of the NF-κB pathway. At the present time, however, we do not have a clear understanding of whether the reduced NF-κB activity was the result of the critical regulation of the transcription factor by M2 macrophages. Reports from several laboratories suggest that macrophages play a role in inducing the activation of NF-κB in epithelial cells in lungs.[44–46] M2 macrophage depletion performed in the present study may have caused an alteration in NF-κB activation in alveolar epithelial cells. Such a regulation may be mediated by soluble mediators, such as TNF-α and IL-1β, because they have been shown to be involved in the macrophage-induced modulation of NF-κB activity in alveolar epithelial cells.[45,46]

Another major proinflammatory transcription pathway within macrophages involves the AP-1 pathway, whose proinflammatory targets overlap those of the NF-κB pathway.[47] AP-1 is a group of basic leucine zipper transcription factors, including the Fos and Jun families of transcription factors.[47] We found that AP-1 c-Jun and c-Fos activities were greatly enhanced when DT was given to CD206-DT receptor Tg mice with endotoxemia, which implies that M2 macrophages negatively regulate the AP-1 pathway. We, thus, suggest that AP-1 signaling is a key transcriptional regulator involved in the inhibitory modulation of lung inflammation by M2 macrophages. There were slight differences in the extents of the enhancing effect of DT treatment on an array of proinflammatory cytokine levels in CD206-DT receptor Tg mice. This may be associated with some differences in the dependence of the transcription of cytokines on the NF-κB and AP-1 pathways. In accord with the importance of AP-1 in regulating *IL-10* promoter activity,[48] a striking up-regulation of IL-10 mRNA was found when CD206-depleted mice were challenged with LPS. However, the increased AP-1 activity may be secondary to the significant result of CD206 depletion leading to altered LPS responses. Further investigations are required to delineate a mechanistic role of AP-1 in the

increased pulmonary inflammatory response in CD206-depleted mice.

We have previously demonstrated that short-term treatment with high doses of statin can increase the number of alveolar macrophages in mouse lungs.[49] These alveolar macrophages display an unusual phenotype compared with typical tissue macrophages. Thus, we have found that alveolar macrophages express high levels of CD11c,[49] a molecule that is not expressed by their counterparts in other body sites and is generally expressed by dendritic cells.[50] However, contrary to dendritic cells, alveolar macrophages are unlikely to emigrate from the tissue and seem to have distinct roles in the initiation and maintenance of immune response.[51] These alveolar macrophages may be now identified as M2 macrophages. Our statin treatment has been able to mitigate ALI and improve the survival of mice with cecal ligation and puncture-induced sepsis.[49]

In conclusion, our findings identify lung CD206-positive M2 macrophages as key anti-inflammatory cells during endotoxemic lung injury. We clearly demonstrate that the depletion of M2 macrophages can aggravate lung inflammation that is linked with neutrophil recruitment to the lung. Our finding may become part of a therapeutic strategy for pulmonary inflammatory disease.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2014.09.005*.

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Increased production of intestinal immunoglobulins in *Syntenin-1*-deficient mice

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ABSTRACT

Syntenin-1 is an intracellular PDZ protein that binds multiple proteins and regulates protein trafficking, cancer metastasis, exosome production, synaptic formation, and IL-5 signaling. However, the functions of Syntenin-1 have not yet been clearly characterized in detail, especially *in vivo*. In this study, we generated a *Syntenin-1* knock out (KO) mouse strain and analyzed the role(s) of Syntenin-1 in IL-5 signaling, because the direct interaction of Syntenin-1 with the cytoplasmic domain of the IL-5 receptor α subunit and the regulation of IL-5 signaling by Syntenin-1 have been reported. Unexpectedly, the number of IL-5-responding cells was normal and the levels of fecal immunoglobulins were rather higher in the *Syntenin-1* KO mice. We also found that IgA and IgM production of splenic B cells stimulated *in vitro* was increased in *Syntenin-1* KO mice. In addition, we showed that a distribution of intestinal microbial flora was influenced in *Syntenin-1* KO mice. Our data indicate that Syntenin-1 negatively regulates the intestinal immunoglobulin production and has a function to maintain the intestinal homeostasis *in vivo*. The analysis of *Syntenin-1* KO mice may provide novel information on not only mucosal immunity but also other functions of Syntenin-1 such as cancer metastasis and neural development.

Introduction

Syntenin-1 (Syndecan-binding protein, Sdcbp) was first identified as an intracellular scaffold protein interacting with the transmembrane heparan sulfate syndecans (Grootjans et al., 1997). It was also termed melanoma differentiation-associated gene-9 (*MDA*-9), which promoted cancer metastasis by regulating cell adhesion (Boukerche et al., 2005, 2007, 2008; Lin et al., 1998). Subsequently, Syntenin-1 has been reported to act as a multifunctional intracellular adapter protein and to regulate protein trafficking and recycling (Zimmermann et al., 2005), Notch signaling (Estrach et al., 2007), IL-5 signaling (Beekman et al., 2009; Geijsen et al., 2001), T cell chemotaxis (Sala-Valdes et al., 2012), HIV infection (Gordon-Alonso et al., 2012), exosome production (Baietti et al., 2012), and synaptic formation in CNS (Hirbec et al., 2005; Jannatipour et al., 2001; Ohno et al., 2004). Additionally, we previously found that extracellular Syntenin-1 in human colostrum could preferentially induce IgA production from cord blood naive B cells (Sira et al., 2009).

IL-5/IL-5R signaling, one of the pathways interacting with Syntenin-1, was reported to maintain mouse B-1 B cells and promote secretion of mucosal IgA (Moon et al., 2004). IL-5 also promotes eosinophil differentiation in humans and mice (Hiroi et al., 1999; Kopf et al., 1996; Moon et al., 2004; Yoshida et al., 1996). Structurally, IL-5R consists of two distinct subunits, an

Abbreviations: BAC, bacterial artificial chromosome; bp, base pair; CNS, central nervous system; ES, embryonic stem; IL-5Rα, interleukin-5 receptor α subunit; KO, knock out; L-LP, large intestinal lamina propria; LP, lamina propria; PC, peritoneal cavity; PDZ, PSD-95/Discs large/zO-1; PEC, peritoneal exudate cells; PP, Peyer's patch; rRNA, ribosomal RNA; sIgA, surface-IgA; S-LP, small intestinal lamina propria; WT, wild type.

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IL-5R-specific α subunit (IL-5Rα) and a common β subunit for the GM-CSF receptor family (Mita et al., 1989). Syntenin-1 was reported to associate with the cytoplasmic tail of IL-5Rα through the PDZ (PSD-95/Discs large/zO-1) domain and to interact directly with the transcription factor Sox4 (Beekman et al., 2009, 2012; Geijsen et al., 2001). Interestingly, Sox4 promotes B cell development, as determined by analysis of *Sox4* KO mice (Schilham et al., 1996; Sun et al., 2013).

Although these various functions of Syntenin-1 *in vitro* have been well reported including the relationship with IL-5R, little is known about the distribution and role of this protein *in vivo*. To clarify the physiological role(s) of Syntenin-1 *in vivo*, we generated a *Syntenin-1* KO mouse strain by gene targeting in this study. We found that *Syntenin-1* KO mice showed no obvious signs of diseases under specific pathogen-free conditions and the Syntenin-1 was widely expressed, particularly in immunologically related organs and CNS. In addition, we focused on the relationship between Syntenin-1 and IL-5 signaling in gut-associated tissues and found that Syntenin-1 was not essential for the maintenance of IL-5-responding cells, and rather negatively regulated immunoglobulin production in the intestine.

Materials and methods

Generation of Syntenin-1 KO mice

Animal care and experimental protocols were approved by the Animal Experiment Committee of the University of Toyama (Authorization No. A2012-MED-35) and were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animal of the University of Toyama.

A bacterial artificial chromosome (BAC) genomic clone (RP24-301N) originating from the DNA of C57BL/6 mice and containing *Syntenin-1* was obtained from BACPAC Resource Center CHORI (Oakland, CA). A counter-selection BAC modification kit (Gene Bridges, Dresden, Germany) and a MultiSite Gateway Three-Fragment Vector Construction kit (Invitrogen, Carlsbad, CA) were modified for the targeting vector construction. The nucleotide sequence of the mouse genome was obtained from the National Center for Biotechnology Information (NCBI Map Viewer, *Mus musculus* Build 37.1) and the initiation site of translation in *Syntenin-1* (the A of ATG) refers to position +1 and the proceeding residues are indicated by negative numbers in this report. The 5′ arm of ~5 kbp (base pair) (Nos. −4769 to −334) and 3′ arm of ~5 kbp (Nos. +322 to +4947) were subcloned into the pDONR P4-P1R and pDONER P2R-P3 vectors, respectively, using the counter-selection BAC modification kit. The 655-bp *Syntenin-1* (Nos. −333 to +321) gene fragment containing exon 2, part of intron 1, and part of intron 2 was amplified by PCR and subcloned between two loxP sequences of a modified pDONR 221 vector containing a pgk-Neo cassette flanked by two FRT sites. To construct the targeting vector, these three plasmids were directionally subcloned into pDEST R4-R3 containing the diphtheria toxin gene (MC1-DTA) by MultiSite Gateway LR recombination reaction. The targeting vector linearized with *Not*I was electroporated into the embryonic stem (ES) cell line RENKA derived from the C57BL/6N strain (Fukaya et al., 2006) as previously described (Miya et al., 2008). After the selection with G418, recombinant ES clone was identified by Southern blot analysis using the 5′ probe (Nos. −5412 to −4923) on *Spe*I-digested genomic DNA, the 3′ probe (+6740 to +7093) on *Apa*I-digested genomic DNA, and the Neo probe (Miya et al., 2008) on *Apa*I-digested genomic DNA. The obtained recombinant ES clone was transfected with the pCre-Pac plasmid (Taniguchi et al., 1998) and pCAGGS-FLP plasmid (Gene Bridges, Dresden, Germany) by electroporation to delete exon 2 and the pgk-neo cassette, respectively. The PCR amplified fragments were verified using the DNA sequencer ABI PRISM 3100 (Perkin-Elmer, Foster City, CA).

The obtained clone was injected into eight-cell stage embryos of the mouse strain ICR. The embryos were cultured to the blastocyst stage and transferred to the pseudopregnant ICR mouse uterus. The resulting male chimeric mice were crossed with female C57BL/6 mice to establish the mutant mouse line. The *Syntenin-1* KO mice were further genotyped by PCR using the following primers; 5′ forward, 5′-TGACCCTGGTTTAGCTGAGGA-3′; 5′ reverse, 5′-TCTGTTCCCACAGCTACCCAA-3′; and 3′ reverse, 5′-GCTCACAACCGTCTAACTCCAAC-3′ (Fig. 1A).

Western blotting

At the age of 6 weeks, wild type (WT) and *Syntenin-1* KO mice were deeply anesthetized with pentobarbital sodium (100 mg/kg body weight, intraperitoneal injection) and then perfused transcardially with ice-cold PBS. Tissues were quickly removed and homogenized in Mammalian Tissue Extraction Reagent (Pierce, Rockford, IL) with Protease Inhibitor (Nacalai, Kyoto, Japan). The homogenate was centrifuged at 14,500 rpm for 15 min to remove large debris. The protein concentration was determined using a BCA Protein Assay kit (Pierce) and the protein samples were diluted at 1:1 in a sample buffer (50 mM Tris–HCl, pH 8.2, 2% SDS, 10% glycerol, 6% 2-mercaptoethanol, and 0.01% bromophenol blue). After denaturation by heating at 95 °C for 5 min, 30 μg of proteins were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Perkin-Elmer). After blocking with 5% skim milk in Tris buffered saline containing 0.1% Tween-20 for 1 h, the membranes were incubated with rabbit polyclonal anti-Syntenin-1 antibody (1:1000, Abcam, Cambridge, UK) or mouse monoclonal anti-β-actin antibody (1:10,000, Sigma–Aldrich, St. Louis, MO) overnight at 4 °C, then with HRP-conjugated goat anti-rabbit IgG (1:25,000, Bio-Rad, Richmond, CA) or goat anti-mouse IgG (1:25,000, Bio-Rad) for 1 h. Protein bands were detected using the ECL chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK).

Antibodies and reagents for flow cytometry

Antibodies used for flow cytometry were anti-mouse B220 (RA3-6B2), CD3ε (145-2C11), CD5 (53–7.3), CD19 (1D3), CD23 (B3B4), and CD45 (30-F11) antibodies purchased from eBioscience (San Jose, CA) and anti-mouse CD21/35 (7G6), surface-IgA (sIgA) (C10-3), and Siglec-F (E50-2440) antibodies purchased from BD Biosciences (San Diego, CA). FcγRs were blocked with anti-mouse FcγR (2.4G2). Flow cytometry was performed using a FACSCanto II (BD Biosciences). Dead cells were gated out by 7-aminoactinomycin D staining (BD Biosciences). FlowJo (Tree Star, Ashland, OR) was used for analysis.

Preparation of lamina propria cells

To obtain lamina propria (LP) cells, the small and large intestines were harvested, and Peyer's patchs (PPs) and cecal patches were removed. The intestines were then opened longitudinally, washed twice with 40 ml of Ca^{2+}- and Mg^{2+}-free HBSS (Sigma–Aldrich) supplemented with 5% FCS, 1 mM DTT, and 5 mM EDTA and then incubated at 37 °C for 40 min with shaking at 150 rpm. Tissues were minced and incubated with RPMI 1640 (Invitrogen) supplemented with 5% FCS. To the small intestine tissues, 1 mg/ml collagenase type I

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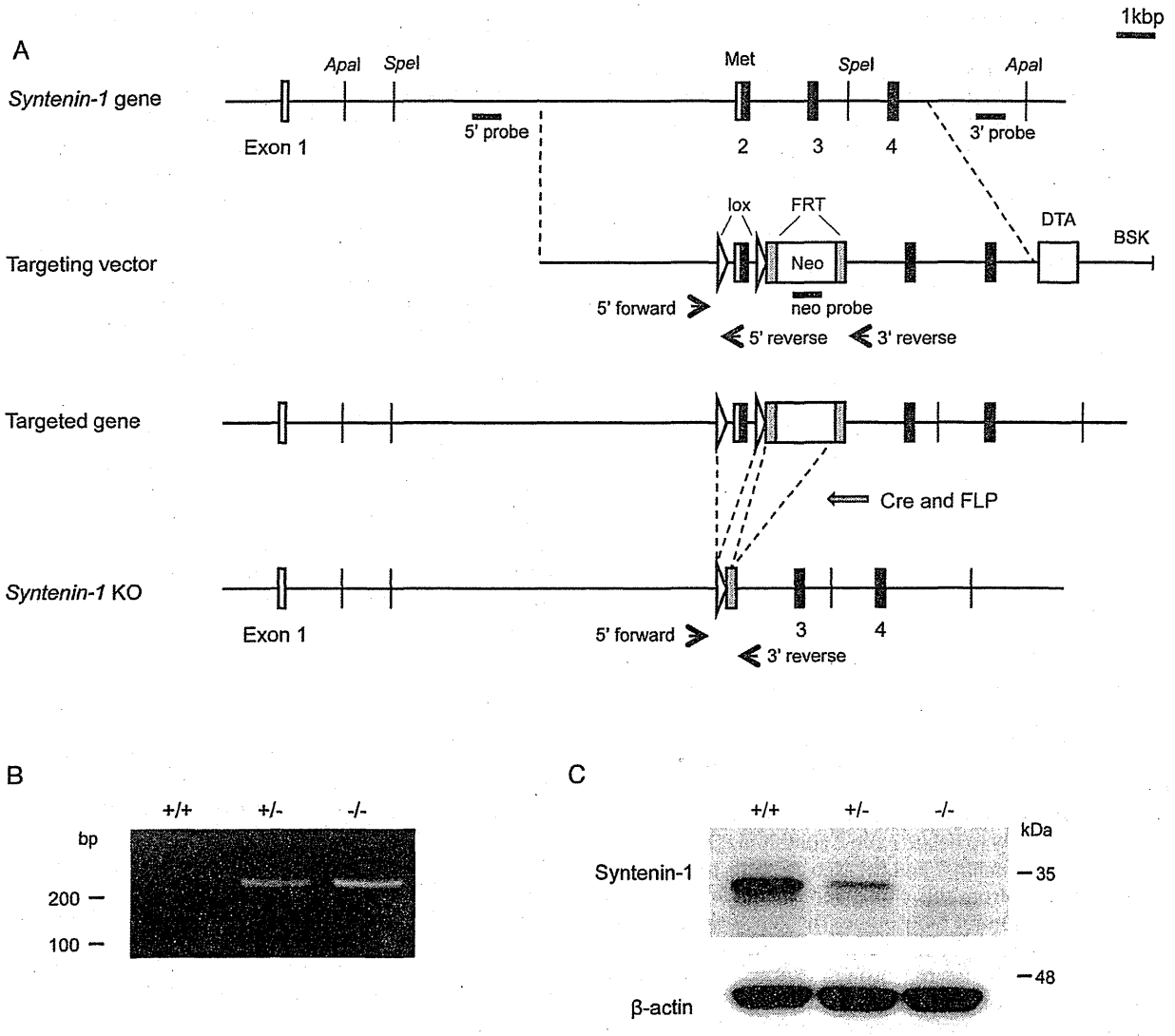

Fig. 1. Generation of *Syntenin-1* KO mice with C57BL/6 genetic background. (A) Schematic representations of *Syntenin-1* gene, targeting vector, targeted gene, and *Syntenin-1* KO gene. The coding and noncoding regions of *Syntenin-1* exons are indicated by closed and open boxes, respectively. Met in exon 2 is the initiation site of translation in *Syntenin-1*. Inserted lox, FRT, and neomycin resistance gene (Neo) are shown. The relevant restriction enzyme sites (*Apa*I and *Spe*I), the location of probes used (5′probe, neo probe, and 3′probe), and the PCR primers (5′forward, 5′reverse, and 3′reverse) are indicated. DTA, diphtheria toxin fragment A; BSK, pBluescript. (B) PCR analysis of genomic DNA from *Syntenin-1*$^{+/+}$ (+/+), *Syntenin-1*$^{+/-}$ (+/−), and *Syntenin-1*$^{-/-}$ (−/−) mice. The 5′forward, 5′reverse, and 3′reverse primers were mixed at a molar ratio of 2:1:1. The positions of DNA size markers are indicated on the left side. (C) Expression of syntenin-1 protein in spleen. Spleen homogenates from *Syntenin-1*$^{+/+}$ (+/+), *Syntenin-1*$^{+/-}$ (+/−), and *Syntenin-1*$^{-/-}$ (−/−) mice were separated by SDS-PAGE and immunoblotted with anti-Syntenin-1 antibody (upper) and anti-β-actin antibody (lower). The positions of protein size markers are indicated on the right side.

(Sigma–Aldrich) was added, and 2 mg/ml collagenase was added to the large intestine tissues. The tissues were then incubated with 100 ng/ml DNase I (Roche Diagnostics, Indianapolis, IN) at 37 °C for 40 min with stirring. Collected cells were placed on the boundary between 40/75% concentrations of Percoll (GE Healthcare, Piscataway, NJ) solution and centrifuged at 1800 rpm at 20 °C for 20 min. After centrifugation, the collected cells were washed and used as LP lymphocytes.

ELISA

Freshly collected fecal samples were weighed, dissolved in PBS (0.1 g/ml), and centrifuged at 15,000 rpm for 5 min. The supernatants were used as fecal extract. The levels of each immunoglobulin isotype in fecal extract and serum were determined by sandwich ELISA using antibodies specific for each murine immunoglobulin isotype (Southern Biotech, Birmingham, AL) according to a protocol.

Splenic B cell purification and cell culture

For collection of resting B cells, single cell suspensions prepared from the spleen isolated from WT and *Syntenin-1* KO mice were purified by magnetic-activated cell sorting (MACS) negative selection using biotin-conjugated anti-mouse CD43 antibody (S7, BD Bioscience) and streptavidin MicroBeads (Miltenyi Biotec, Auburn, CA). The purified resting B cells were cultured at a concentration of 2×10^5 cells/well in RPMI 1640 with 10% FCS, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. 1 μg/ml LPS (Sigma–Aldrich), 1 ng/ml TGF-β (R and D Systems, Minneapolis, MN), and/or 5 ng/ml IL-5 (R and D Systems) were added to the culture to induce IgA production. 0.1 or 1 μg/ml LPS (Sigma–Aldrich) were used to induce IgM production. 1 μg/ml anti-CD40 antibody (R and D Systems) and 50 ng/ml IL-4 (R and D Systems) were added to induce IgG1 production. The concentration of IgA, IgM and IgG1 in the supernatants was measured by ELISA on day 7, day 5 and day 5, respectively.

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DNA extraction from fecal samples and real-time PCR

Fecal samples were collected for 24 h from individually housed *Syntenin-1* KO and WT littermates (6 weeks of age) and stored at −20 °C until analysis. DNA extraction was performed on the fecal samples using DNA stool mini kit (Qiagen, Venlo, The Netherlands). Quantitative real-time PCR assays of 16S ribosomal RNA (rRNA) gene were performed by Cosmobio Co. (Sapporo, Japan) using the method of Matsuki et al. (2002, 2004). Three targets were analyzed: all bacteria, phylum *Firmicutes* and *Bacteroidetes*. The relative ratio of each phylum to all bacteria was measured.

Statistical analysis

All values are represented as mean ± SD. The statistical significance of difference between WT and *Syntenin-1* KO mice was determined by two-tailed Student's t test. Values of $p < 0.05$ indicate a statistically significant difference.

Results

Generation of Syntenin-1 KO mouse strain

To disrupt the *Syntenin-1* locus in ES cells derived from the C57BL/6 mouse strain, we constructed a targeting vector to introduce the loxP sequence into intron 1 and another loxP and the Neo cassette flanked with FRT sequences into intron 2 (Fig. 1A). We obtained an ES cell clone in which the expected homologous recombination occurred at the *Syntenin-1* locus, as detected by Southern blot analysis. The ES cells were treated with Cre and FLP recombinases transiently to delete exon 2 containing the initiation site of translation of *Syntenin-1* and the Neo cassette, respectively. Chimeric mice derived from this clone were mated with C57BL/6 mice to establish the mutant mouse line. The gene deletion in the mutant mice was confirmed by Southern blot (data not shown) and PCR analyses (Fig. 1B). Syntenin-1 protein expression was examined using the homogenate of spleen by Western blot analysis. The rabbit anti-Syntenin-1 antibody recognized a protein detected as a band of 32 kDa corresponding to Syntenin-1 in the WT mice, whereas the band showed decreased intensity in the case of heterozygous mutant mice, and not detected in homozygous mutant mice (Fig. 1C). These findings indicate that *Syntenin-1* was successfully disrupted in the mutant mice.

Mice lacking Syntenin-1 were born at the expected Mendelian ratio (Supplementary Table 1). The *Syntenin-1* KO mice thrived and reproduced as well as their WT littermates and showed no obvious signs of diseases under specific-pathogen free conditions during the first 1 year of life.

Syntenin-1 protein was widely expressed, especially in immunologically related organs and CNS

To evaluate the detailed expression pattern of the Syntenin-1 protein *in vivo*, equal amounts of the protein from various organs of WT mice were examined by Western blotting (Fig. 2). The Syntenin-1 protein was widely expressed in mouse tissues, and relatively high expression levels were detected in the spleen, thymus, and brain. The Syntenin-1 expression levels in the liver and kidney were lower than those in the other tissues. We used lysates of human embryonic kidney (HEK) 293 cells transfected with a human *Syntenin-1* expression plasmid as a positive control. The Syntenin-1 protein was not detected in any organs of *Syntenin-1* KO mice (Fig. 2 and data not shown), suggesting the specificity of the primary antibody used against Syntenin-1.

The number of IL-5-responding cells was not affected in Syntenin-1-deficient mice

IL-5 signaling is a key regulator for the maintenance of B-1 B cells, IgA production, and eosinophils in mice (Hiroi et al., 1999; Moon et al., 2004; Tominaga et al., 1991). To clarify whether Syntenin-1 is involved in the development and maintenance of the IL-5-responding cells, we examined the number of lymphocytes and eosinophils in the peritoneal cavity (PC), mesenteric lymph node (MLN), PP, and small and large intestinal lamina propria (S-LP and L-LP, respectively). The proportions of $B220^+$ $sIgA^-$, $B220^+sIgA^+$, and $B220^-sIgA^+$ B cells in the S-LP and L-LP and $B220^+sIgA^-$ and $B220^+sIgA^+$ B cells in PP and MLN, as well as $CD3\varepsilon^+$ T cells, were normal in *Syntenin-1* KO mice (Supplementary Fig. 1A). Siglec-F^+ eosinophils in the S-LP and L-LP of *Syntenin-1* KO mice also normally developed (Supplementary Fig. 1B). B-1a and B-1b cells (characterized as $CD19^+CD21^-CD23^-CD5^+$ and $CD19^+CD21^-CD23^-CD5^-$, respectively) and conventional B-2 cells in PC seemed to be not affected by Syntenin-1 deficiency (Supplementary Fig. 1C). The number of the analyzed immune cells showed no significant differences between *Syntenin-1* KO and WT mice (Table 1). These results suggest that Syntenin-1 little affected on the development of lymphocytes and eosinophils in the steady state.

Production of fecal immunoglobulins were increased in Syntenin-1-deficient mice

Given that IL-5 is a key cytokine for IgA production, isotype-specific ELISA was performed to determine levels of IgA together with those of IgG1 and IgM in the fecal extract and serum in *Syntenin-1* KO and WT mice. Although the levels of immunoglobulins in the serum did not change, IgA, IgG1 and IgM levels in the fecal extract increased significantly in *Syntenin-1* KO mice (Fig. 3Aand B). These results showed that Syntenin-1 negatively regulates immunoglobulin production in the intestine.

In vitro analysis of immunoglobulins production

As intestinal immunoglobulin production was increased in *Syntenin-1* KO mice, we next examined the ability of B cells to secrete immunoglobulins in WT and *Syntenin-1* KO mice *in vitro*. We purified splenic resting B cells by magnetic cell isolation and cultured them in the presence of LPS, TGF-β, and IL-5 to induce IgA secretion; LPS to induce IgM secretion; IL-4 and anti-CD40 antibody to induce IgG1 secretion. IgA production was significantly higher in B cells derived from *Syntenin-1*-deficient mice than in B cells derived from WT mice (Fig. 4A). IgM production of *Syntenin-1* KO B cells stimulated with only LPS was also increased (Fig. 4B). IgG1 production had no significant difference between these mouse strains (Fig. 4C). These results support the idea that Syntenin-1 negatively regulates immunoglobulin production in the intestine and suggest that such an enhanced immunoglobulin production occurs in a B cell-intrinsic manner.

Distribution of intestinal microbiota was influenced in Syntenin-1-deficient mice

Mucosal immunity including intestinal secretory immunoglobulins is closely related to microbiota to maintain intestinal homeostasis (Strugnell and Wijburg, 2010). The obtained results in *Syntenin-1* KO mice raise a question whether a distribution of intestinal microbial flora could be influenced. We next examined the percentages of total 16S rRNA gene of the phylum *Firmicutes* and *Bacteroidetes*, which consist mostly of mouse intestinal microbiota. The percentage of *Firmicutes* in the stool of *Syntenin-1* KO

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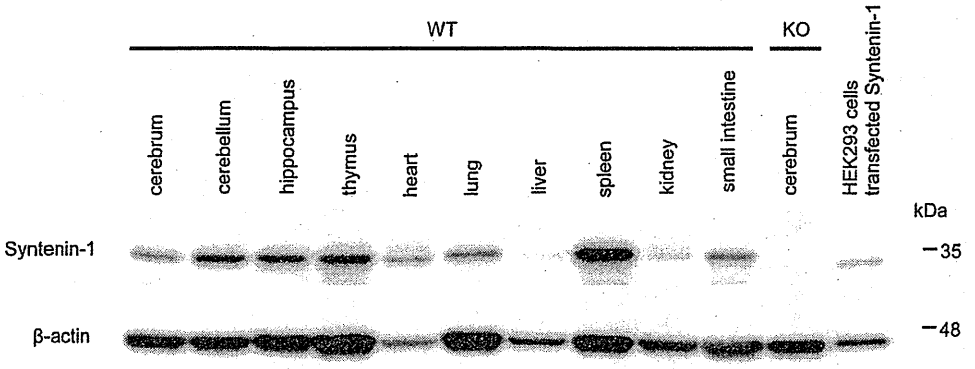

Fig. 2. Expression pattern of syntenin-1 protein. Western blot analysis of protein extracts from cerebrum, cerebellum, hippocampus, thymus, heart, lung, liver, spleen, kidney, and small intestine in WT mice and cerebrum in *Syntenin-1* KO mice using anti-Syntenin-1 antibody (upper panels) and anti-β-actin antibody (lower panels). Equal amounts (30 μg) of protein measured by BCA protein assay were loaded to all lanes. Lysate of human embryonic kidney (HEK) 293 cells (5 μg) transfected with the human *Syntenin-1* expression plasmid was used as a positive control. The positions of protein size markers are indicated on the right side. Data are representative of at least three independent experiments.

mice was significantly higher than WT mice, although the percentage of *Bacteroidetes* and the ratio of *Firmicutes* to *Bacteroidetes* were not significantly different (Fig. 5).

Discussion

We have generated a *Syntenin-1* KO mouse strain to clarify the function(s) of Syntenin-1 *in vivo*. Although Syntenin-1 was widely expressed in mouse organs, homozygous *Syntenin-1*-deficient mice showed no obvious signs of diseases. We found that the levels of fecal immunoglobulins in *Syntenin-1* KO mice were higher than those in WT mice. We also showed that the levels of IgA and IgM secretion from LPS-stimulated splenic B cells were significantly higher in *Syntenin-1* KO mice, therefore the mechanism underlying enhanced fecal immunoglobulins production in *Syntenin-1* KO mice is likely B cell-intrinsic. Additionally, we indicated a possibility that the intestinal microbiota was influenced in *Syntenin-1* KO mice. Taken together, our results imply that Syntenin-1 has a function to maintain the intestinal homeostasis *in vivo*.

Structurally, Syntenin-1 is a 32-kDa protein with two PDZ domains (Das et al., 2012). The PDZ domains can bind to short amino acid sequences at the C-terminal end of the transmembrane or intracellular proteins (Chimura et al., 2011). Through the PDZ domains, Syntenin-1 is capable to bind to various proteins, such as syndecans, the tyrosin kinase Src, IL-5Rα, CD63, Delta1, and adhesion molecules, for synaptic formation in the CNS (Boukerche et al., 2008; Estrach et al., 2007; Geijsen et al., 2001; Grootjans et al., 1997; Hirbec et al., 2005; Jannatipour et al., 2001; Ohno et al., 2004; Pols and Klumperman, 2009). In general, several PDZ proteins are reported to act as a negative regulator of various signals and transcription factors (Alewine et al., 2006; Gupta et al., 2012; Stephenson et al., 2007). In fact, Chen et al. reported that Syntenin specifically interacted with TNF receptor associated factor 6 (TRAF6) and played inhibitory role in TLR4-mediated NF-kB activation signaling pathway (Chen et al., 2008). Therefore, Syntenin-1 could potentially inhibit the signaling pathway of immunoglobulin production.

Although the interacting partners of Syntenin-1 were well reported, little has been known about the distribution of the Syntenin-1 protein *in vivo*. Jeon et al. analyzed the expression pattern of the Syntenin protein in mouse embryos by immunohistochemistry (Jeon et al., 2013). They reported that the Syntenin protein was detected temporally during an early developmental period, and that Syntenin may play a prominent role in cell proliferation and differentiation in normal mouse development. However, in this study we found that the Syntenin-1 protein was widely expressed in adult mouse organs, especially in the spleen, thymus, and brain. Our expression data are consistent with various reports which describe the functions of Syntenin-1 in the immune system and CNS (Gordon-Alonso et al., 2012; Jannatipour et al., 2001; Koroll

Table 1
Numbers of sIgA$^+$ cells, T cells, eosinophils, and B-1a, B-1b, and B-2 cells in gut-associated lymphoid tissues and peritoneal cavity.

Syntenin-1		S-LP	L-LP	MLN	PP	PC
	B220$^+$sIgA$^-$	1.4 ± 0.3	3.0 ± 0.9	11.1 ± 3.2	8.3 ± 2.0	
	B220$^-$sIgA$^+$	19.6 ± 8.2	1.1 ± 0.4			
	B220$^+$sIgA$^+$	0.1 ± 0.1	0.1 ± 0.1	1.0 ± 0.3	1.0 ± 0.4	
	CD3ε$^+$T cell	5.4 ± 3.3	1.3 ± 0.6	22.4 ± 9.5	2.3 ± 0.5	
−/−	Eosinophil	2.0 ± 1.8	0.2 ± 0.1			
	B-1a					2.9 ± 1.5
	B-1b					0.5 ± 0.2
	B-2					5.8 ± 1.1
	B220$^+$sIgA$^-$	1.7 ± 1.1	2.6 ± 1.0	11.1 ± 7.1	10.5 ± 3.3	
	B220$^-$sIgA$^+$	19.6 ± 10.3	1.1 ± 0.4			
	B220$^+$sIgA$^+$	0.1 ± 0.1	0.1 ± 0.0	1.1 ± 0.4	1.3 ± 0.5	
+/+	CD3ε$^+$T cell	5.2 ± 1.7	1.4 ± 0.7	20.7 ± 13.2	2.7 ± 1.2	
	Eosinophil	1.8 ± 1.3	0.3 ± 0.2			
	B-1a					4.7 ± 1.5
	B-1b					0.9 ± 0.4
	B-2					6.0 ± 3.0

The results indicate the mean cell numbers ± SD (×10^5) calculated on the basis of Supplementary Fig. 1 (n = 5 for each group). There were not significantly different between *Syntenin-1* KO mice and WT mice. S-LP, small intestinal lamina propria; L-LP, large intestinal lamina propria; MLN, mesenteric lymph node; PP, Peyer's patch; PC, peritoneal cavity; sIgA, surface-IgA.

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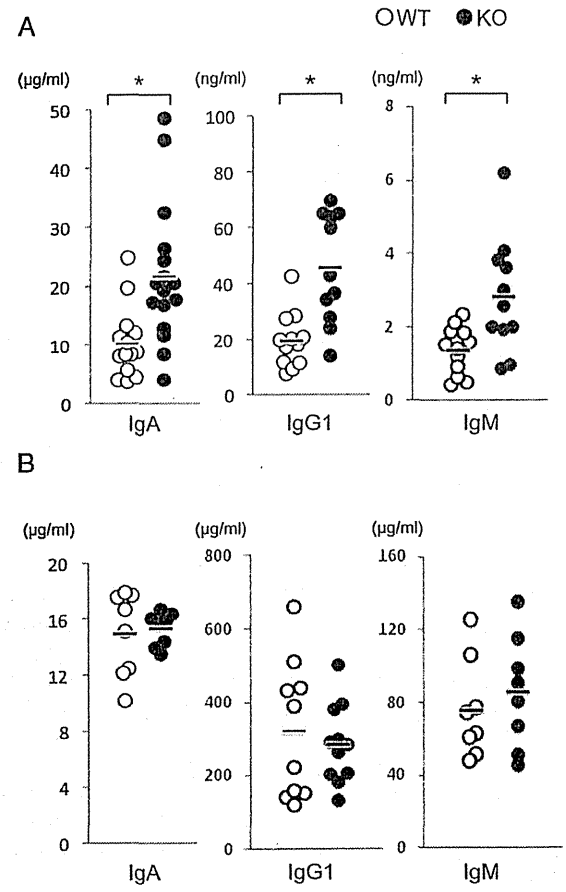

Fig. 3. Immunoglobulin levels in the fecal extract and serum of *Syntenin-1* KO mice. The concentrations of IgA, IgG1, and IgM in fecal extracts (A) and serum (B) in WT (○) and *Syntenin-1* KO (●) mice were determined by isotype-specific ELISA. Each spot represents an individual mouse (6–8 weeks old). The mean levels of immunoglobulins are presented as bars. Asterisks indicate statistically significant differences (*$p < 0.05$), as calculated by two-tailed Student's *t* test.

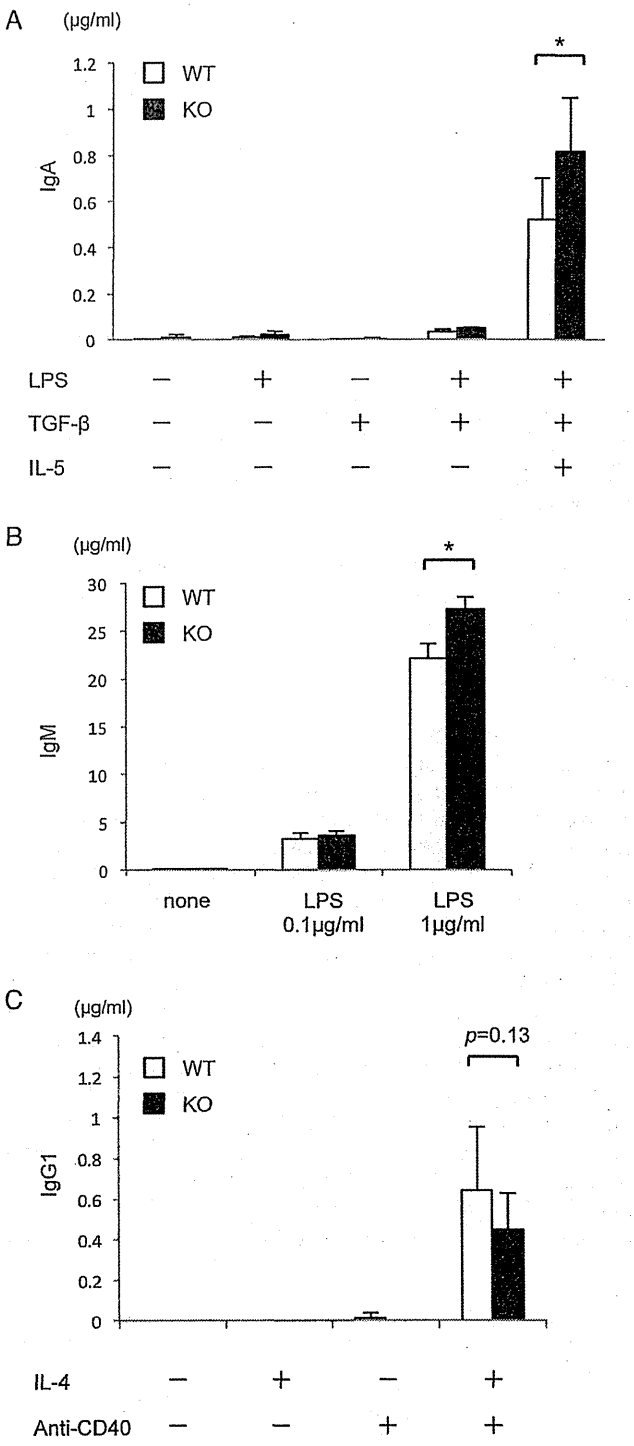

Fig. 4. Increased levels of IgA and IgM secretion from B cells obtained from *Syntenin-1* KO mice *in vitro*. Purified splenic resting B cells from WT (□) and *Syntenin-1* KO (■) mice were cultured with LPS, TGF-β and/or IL-5 for 7 days to induce IgA secretion (A), LPS for 5 days to induce IgM secretion (B), and IL-4 and anti-CD40 antibody for 5 days to induce IgG1 secretion (C). The levels of immunoglobulins in the supernatants were measured by ELISA. Data are shown as mean ± SD and are representative of two or three independent experiments performed in triplicate. Asterisks indicate statistically significant difference (*$p < 0.05$), as calculated by two-tailed Student's *t* test.

et al., 2001; Sala-Valdes et al., 2012) and imply that Syntenin-1 may play important roles in these organs.

Despite the broad expression of the Syntenin-1 protein in all the organs analyzed, the homozygous deletion of *Syntenin-1* was not lethal, and the *Syntenin-1* KO mice showed no obvious signs of diseases under specific pathogen-free conditions. It is possible that some redundant proteins may provide compensatory signals to maintain the homeostasis *in vivo*. In mice and humans, Syntenin has two isoforms, Syntenin-1 and Syntenin-2. Syntenin-2 shares 61% identity in amino acid sequence with Syntenin-1 in mice. Although little is known about the functions of Syntenin-2, it is possible that the presence of Syntenin-2 could compensate for the functions of Syntenin-1 in *Syntenin-1* KO mice. Dual deletion of those *Syntenin* genes might reveal their roles in immunity.

In this study, we focused on the relationship between Syntenin-1 and IL-5 signaling. IL-5 is one of the key regulators in mucosal immunity in mice, especially the development of B-1 B cells and IgA-producing cells and the production of intestinal IgA (Hiroi et al., 1999; Moon et al., 2004; Tominaga et al., 1991). Because Syntenin-1 is reported to interact with IL-5Rα *in vitro* and may affect IL-5 signaling (Geijsen et al., 2001), we investigated the role(s) of Syntenin-1 in gut-associated lymphoid organs. However, the lymphocyte populations in gut-associated lymphoid tissues were not significantly different between *Syntenin-1* KO mice and WT mice, and we found that the levels of immunoglobulins in fecal extract were higher in *Syntenin-1* KO mice. These results suggest that Syntenin-1 plays only minor roles in the IL-5/IL-5R pathway in the mucosal immune system and rather negatively regulates immunoglobulin production in the intestine.

We showed that the IgA and IgM production from stimulated spleen B cells increased in *Syntenin-1* KO B cells. Under physiological conditions, intestinal B cells differentiate into plasma cells via

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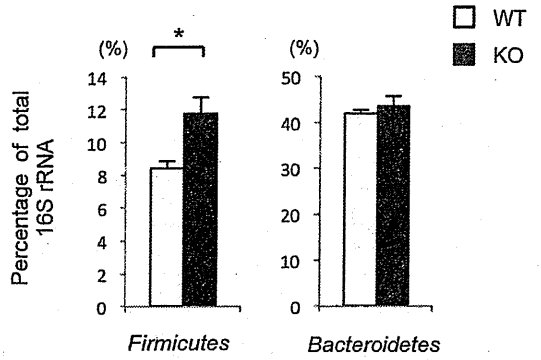

Fig. 5. Percentage of total 16S rRNA gene of phylum *Firmicutes* and *Bacteroidetes* in stool from individually housed *Syntenin-1* KO (■) and WT (□) lettermate mice ($n=5$). Quantitative real-time PCR assays of 16S ribosomal RNA (rRNA) gene were performed by using total bacterial primers and phylum-specific primers. The relative ratio of each phylum to total bacteria was measured. Data are shown as mean ± SD and an asterisk indicates statistically significant differences ($^*p<0.05$), as calculated by two-tailed Student's *t* test.

T cell-dependent and T cell-independent pathways, and secreted immunoglobulins at LP are then transported to the lumen by immunoglobulin receptors (Strugnell and Wijburg, 2010; Horton and Vidarsson, 2013). We showed the lymphocyte populations including intestinal B cells and $B220^{-}IgA^{+}$ cells (which represent plasma cells) were not significantly different and IgA secreting cells in LP were not also different by using ELISPOT assays (data not shown). Although the precise mechanisms were not delineated in this study, our *in vitro* results indicate that such an enhanced immunoglobulin production in the intestine is caused by excessive immunoglobulin production of individual *Syntenin-1*-deficient plasma cells.

Mucosal IgA plays crucial roles in host defense and maintenance of normal gut microbiota (Fagarasan et al., 2002; Horton and Vidarsson, 2013; Strugnell and Wijburg, 2010; Suzuki et al., 2004). Additionally, recent studies have shown that mucosal IgM and IgG can also provide humoral protection from various pathogens (Horton and Vidarsson, 2013; Saeland et al., 2003; Stapleton et al., 2011). On the other hand, mucosal IgG or IgM production is excessively increased in patients with an inflammatory bowel disease such as ulcerative colitis and Crohn's disease (Helgeland et al., 1992; Macpherson et al., 1996; Thoree et al., 2002). In this manner, the relationship between host immunity and intestinal microbiota is essential to maintain homeostatic balance in the gut. In this study, we showed that a distribution of intestinal microbiota in *Syntenin-1* KO mice could be influenced at the phylum level. Although the detailed analysis at the genus level was not examined in this study, it is possible that Syntenin-1 is involved in the maintenance of normal intestinal microbiota and mucosal immune balance by regulating immunoglobulin production.

Previously, we demonstrated that Syntenin-1 exists in the human colostrum and could induce IgA production from naive B cells (Sira et al., 2009). In the present study, we found the negative regulation of Syntenin-1 in IgA production. These incompatible functions of Syntenin-1 may be due to differences among species or effector sites. As our present data might be influenced by the function of extracellular Syntenin-1 in milk, we analyzed littermate offspring from heterozygous breeding pairs to exclude such differences in components of milk in this study. Further studies are necessary to clarify the role and mechanisms of actions of Syntenin-1 in the colostrum *in vivo*.

In summary, in this article we described the generation of *Syntenin-1* KO mice and provided new evidence that Syntenin-1 negatively regulates immunoglobulin production in the intestine. Although Syntenin-1 has been reported to have various functions *in vitro*, additional studies are needed to clarify the mechanisms in detail, especially *in vivo*. Through future studies of *Syntenin-1* KO mice, it will be possible to provide novel evidence of the involvement and functions of Syntenin-1 not only mucosal immunity but also cancer metastasis, protein recycling, exosome formation, and neural network formation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2014.12.003.

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特集・第55回日本小児神経学会学術集会
シンポジウム10：難治性てんかんの病態を探る：分子遺伝学，病理，免疫，代謝異常，画像，電気生理

難治性てんかんの病態を探る

—脳炎後てんかんと免疫

髙橋 幸利　山口 解冬

要旨　代表的な難治性てんかんとして，脳炎後てんかんの臨床，免疫，生化学的特徴を検討した．加療中の症例では，発作頻度は月単位で，知的障害，精神障害などを併存する症例が多く，脳炎急性期から数年経過した時期においても，発作・知的障害が進行悪化すると推測した．局在関連性てんかんでは髄液 NMDA 型 GluR 抗体が高値で，NMDA 型 GluR 内在化，アポトーシス誘導作用等により，病態に影響していると推測され，matrix metalloproteinase-9 の増加，tissue inhibitor of metalloproteinase-1 の減少による血液脳関門障害も病態に影響していると推測された．

見出し語　脳炎後てんかん，NMDA 型 Glutamate receptor 抗体，アポトーシス，血液脳関門，matrix metalloproteinase-9

はじめに

英国での 9 ～ 93 歳の 525 連続てんかん症例の発作予後調査では，63%が発作抑制され，6.5%が治療終結していたが，37%は難治に発作が継続していたとされ，てんかん症例の約 40%が難治性てんかんと推定される[1]．1989 国際てんかん分類でみると，West 症候群，症候性局在関連性てんかん，Lennox-Gastaut 症候群などで発作抑制に至れない難治性てんかん症例の比率が高く，2010 年 ILAE てんかん分類提案で見てみると，Dravet 症候群，海馬硬化症を伴う内側側頭葉てんかん，Rasmussen 症候群，進行性ミオクローヌスてんかんなども難治性てんかんとなりやすい．1993 ～ 1994 年に当センターに入院した小児てんかん症例の集計では，症候性局在関連性てんかんが 50.9%，症候性全般てんかんが 35.2%で，数としては局在関連性てんかんが難治てんかんの多数を占め，原因としては脳炎や脳形成異常，周産期障害などが多い．その中で最も多かった脳炎（脳症を含む）を原因とすると推測されるてんかん症例で，難治化の要因について免疫，生化学的因子を主体に検討した．

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Ⅰ　急性脳炎から脳炎後てんかん

我々の研究班（厚生労働科学研究，こころの健康科学研究事業）の後方視的調査では，日本では 3,100 人／年の急性脳炎発病があり，年齢別にみると 1 ～ 10 歳が多数を占めた．ミネソタでの調査では 7.4 人／年/10 万人の発病率で，5 ～ 9 歳と 1 歳未満が多く，日本に当てはめると 7,500 人／年の脳炎発病があることになる[2]．台湾での 0 ～ 17 歳の急性脳炎 330 例の後方視的調査では，16.4%（54/330）がてんかんを発病し，79.6%は脳炎から 6 カ月以内にてんかんと診断されていた[3]．脳炎急性期に，繰り返す発作，てんかん重積，重症意識障害，限局性の神経学的兆候が見られると，てんかんを発病しやすいと報告されている．脳炎後てんかんのてんかん原性メカニズム，発作原性メカニズムはいまだ未解明で，研究の進展が待たれる．

Ⅱ　脳炎後てんかんの特徴

1. てんかん分類

当センターにおいて筆頭著者が診療した連続症例（初診＋再診，2002 年 4 月～ 2010 年 3 月）で小児期発病のてんかん 586 例について，てんかんの病因と 1989 国際てんかん分類の関係を検討すると，脳炎によるてんかんでは，染色体異常（Fisher's exact test，$p=0.0005$），遺伝子異常（$p=0.0436$），皮質形成異常（$p=0.0263$），仮死（$p=0.0081$）によるてんかんに比べて，West 症候群，Lennox-Gastaut 症候群などの乳児てんかん性脳症（EE）の頻度が有意に低い（図 1）．また，脳炎後てんかんは，染色体異常（Fisher's exact test，$p=0.0079$），遺伝子異常（Fisher's exact test，$p<0.0001$）に比べて，有意に

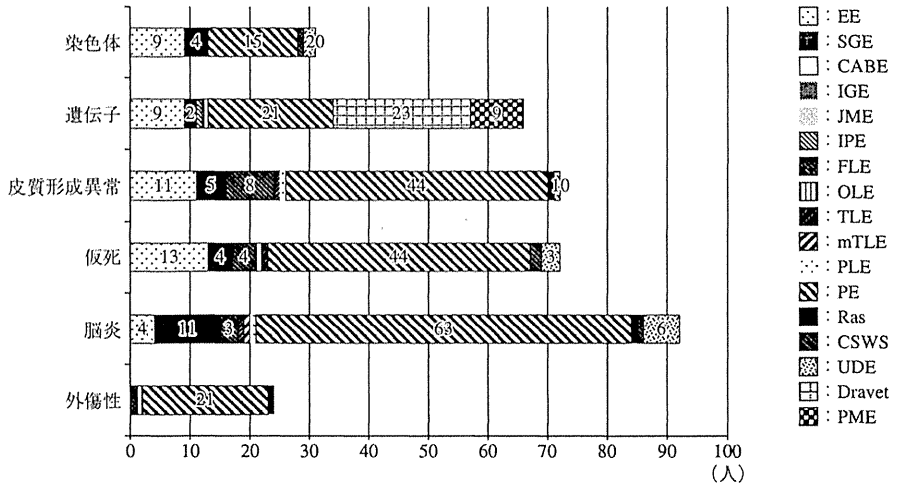

図1 小児難治性てんかん586例の病因とてんかん分類

横軸およびカラム内の数字は症例数を示す.

EE:epileptic encephalopathy (West syndrome, EIEE, Lennox-Gastaut syndrome, etc.), SGE:symptomatic generalized epilepsy, CABE:childhood absence epilepsy, IGE:idiopathic generalized epilepsy, JME:juvenile myoclonic epilepsy, IPE:idiopathic partial epilepsy, FLE:frontal lobe epilepsy, OLE:occipital lobe epilepsy, TLE:temporal lobe epilepsy, mTLE:mesial temporal lobe epilepsy, PLE:parietal lobe epilepsy, PE:partial epilepsy, Ras:Rasmussen syndrome, CSWS:epilepsy with continuous spike and wave complex during slow wave sleep, UDE:undetermined epilepsy, Dravet:Dravet syndrome, PME:progressive myoclonus epilepsy.

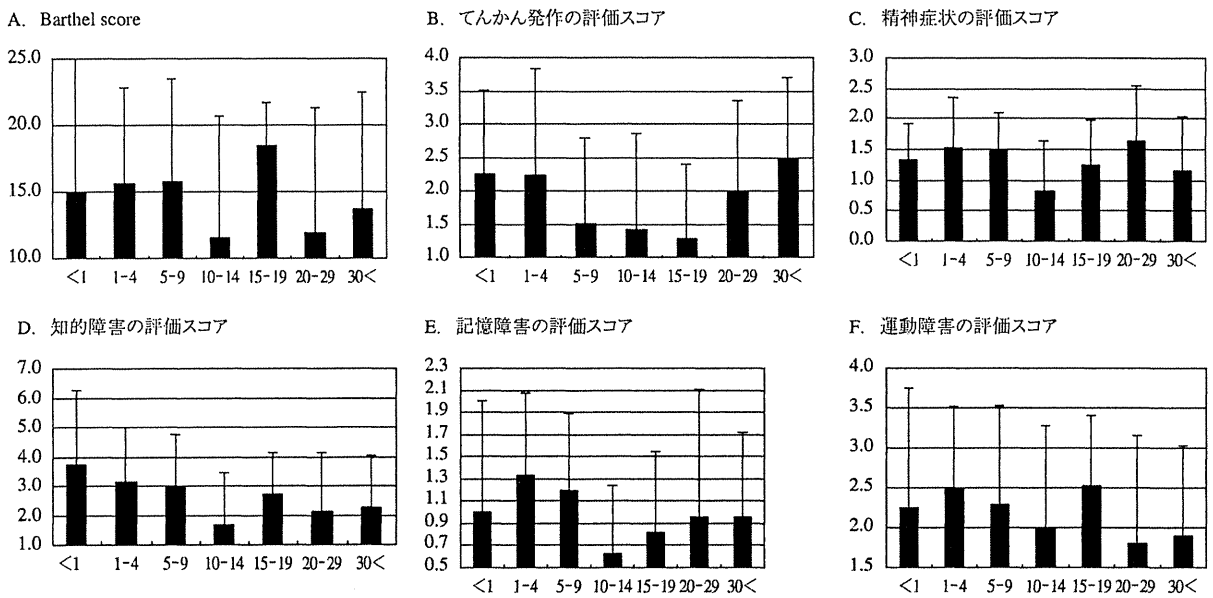

図2 脳炎後の罹病期間と脳炎後遺症

脳炎後てんかん慢性期199例の脳炎後の罹病期間と後遺症の程度を示す.

横軸は脳炎後の罹病期間(年)を,カラムは平均+SDを示す.A:ADLはBarthel score (http://www.patient.co.uk/printer.asp?doc=40001654)に基づいて20点満点で評価した.B:てんかん発作の予後は,発作頻度によりスコア0(日単位),1(週単位),2(月単位),3(年単位),4(抑制)に分類,C:精神障害は0(精神症状のため日常生活が自立困難),1(精神症状はあるが日常生活は自立可能),2(精神症状はない)に,D:知的障害はIQまたはDQによりスコア0(IQ/DQ<19),1(IQ/DQ=34-20),2(IQ/DQ=49-35),3(IQ/DQ=69-50),4(IQ/DQ=79-70),5(IQ/DQ≧80)に,E:記憶障害はスコア0(記憶障害のため日常生活が自立困難),1(記憶障害はあるが日常生活は自立可能),2(記憶障害はない)に,F:運動障害はスコア0(四肢麻痺),1(障害があるが自力移動可能),2(支えなく歩行できるが走れない),3(運動障害はない)に後遺症の程度を分類した.

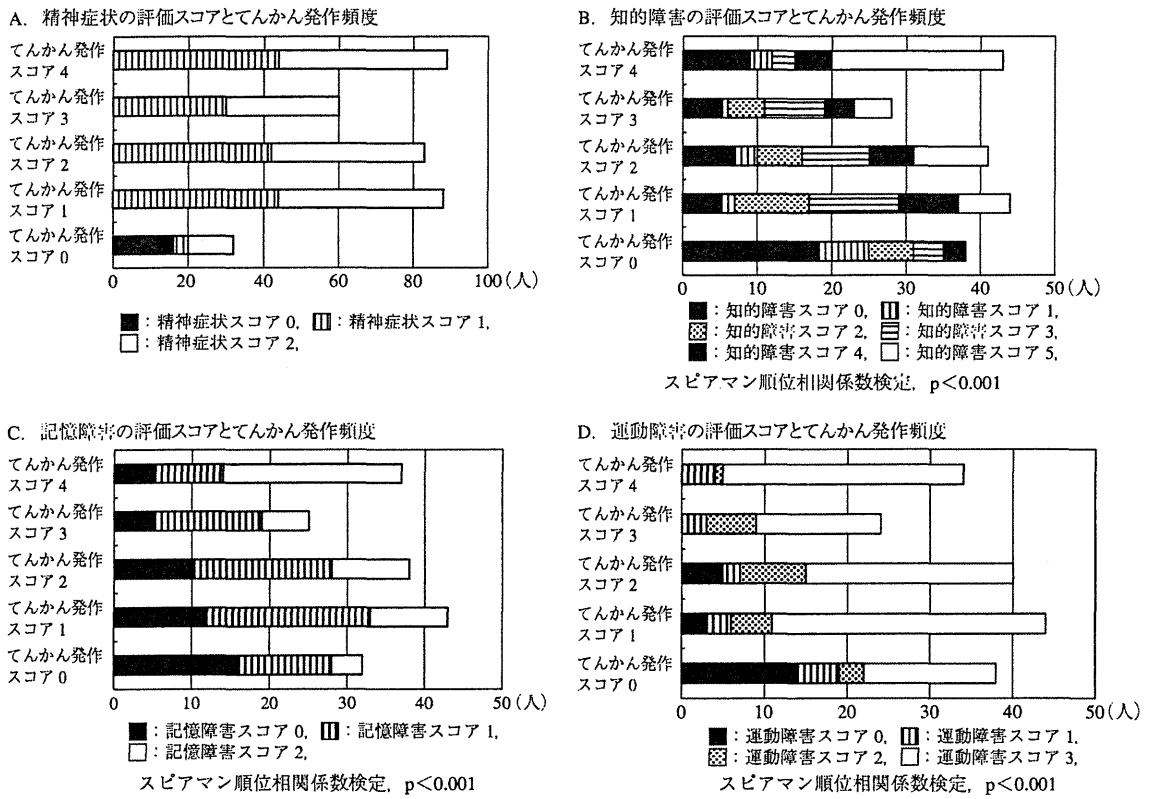

図 3　てんかん発作頻度と併存症

脳炎後てんかん慢性期 199 例のてんかん発作頻度と併存症の程度の関係を示す．
てんかん発作頻度等の予後スコアは図 2 を参照．

症候性局在関連性てんかん（FLE＋OLE＋TLE＋mTLE＋PLE＋PE）の頻度が高い．この傾向は 1 歳未満で発病した症例に限っても同様に見られ，脳炎後てんかんは発病年齢とは無関係に，局在関連性てんかんになりやすいと推定された．

2. 併存症，経過

国立病院機構の病院で加療中の脳炎後てんかんの 199 症例（男＝105 例，女＝94 例）の予後を調査した[4]．対象の急性脳炎（脳症）発病年齢（平均±SD）は 9.3±12.4 歳，調査時年齢は 26.3±16.4 歳，脳炎後の罹病期間は 17.3±16.0 年である．ADL の予後を示す Barthel score は 14.5±8.1（20 点満点），てんかん発作予後は 2.0±1.4（月単位の発作頻度），精神症状予後は 1.4±0.8（軽度障害），知的障害予後は 2.6±1.9（軽～中等度障害），記憶障害予後は 1.1±0.8（軽度障害），運動障害予後は 2.2±1.2（歩行はできるが何らかの障害あり）であった．加療中の脳炎後てんかん症例では，発作が月単位と多く，知的障害，精神障害，運動機能障害などの併存症を有する症例が多かった．知的・運動障害は発病年齢が若いほど強く，年齢に依存した障害メカニズムが存在する可能性が強い．てんかん発作・知的障害は脳炎急性期から数年経過しても慢性期に進行悪化する経過を示唆した（図 2）．てんかん発作の頻度が高いほど，知的障害が強く（スピアマン順位相関係数検定，p<0.001），運動障害の程度も強く（p<0.001），記憶障害も強いことが分かった（p<0.001）（図 3）．このことはてんかん発作の持続が 2 次的に新たな障害を生み出している可能性（てんかん性脳症）を示唆した．我々は，てんかん発作による神経細胞死がグルタミン酸受容体（GluR）などの抗原を放出し，後述する GluR 抗体を増加させ，新たな神経障害を誘導している可能性を推測している．

3. 薬理学的特徴

脳炎以外を病因とする（非脳炎）局在関連性てんかん症例では，topiramate（TPM）の投与量が増えるほど発作減少率が増加する通常の薬理特性が見られたが，脳炎による局在関連性てんかん症例では，TPM の投与量が増えるほど発作減少率が低下する傾向が見られた（図 4）．Lamotrigine については脳炎を病因とする局在関連性てんかん症例とそれ以外の病因の症例で投与量-有効性関係に違いは見られなかった．このように脳炎という病因が投与量-有効性の薬理学的関係に変化をもたらす抗てんかん薬があることが明らかになった．

4. 自己抗体の関与

我々は N-methyl-D-aspartate（NMDA）型 GluR（NR）のサ

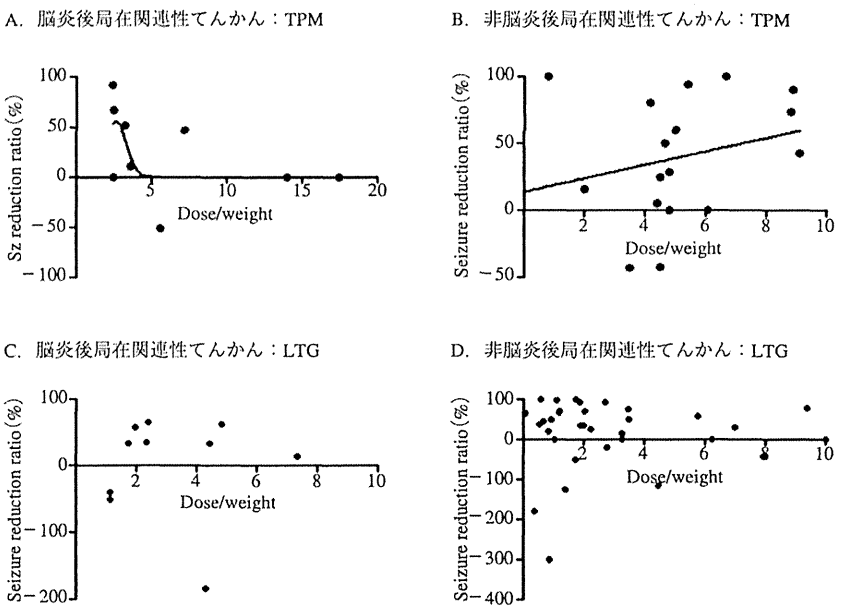

図4 Topiramate（TPM）およびlamotrigine（LTG）の投与量－有効性関係

A：TPMの脳炎後局在関連性てんかん（小児＋成人11例）における投与量－有効性関係，B：TPMの非脳炎後局在関連性てんかん（小児41例）における投与量－有効性関係，C：LTGの脳炎後局在関連性てんかん（小児＋成人17例）における投与量－有効性関係，D：LTGの非脳炎後局在関連性てんかん（小児57例）における投与量－有効性関係．

横軸は体重あたりの投与量（mg/kg），縦軸は発作減少率（Seizure reduction ratio）（%）＝(投与前発作頻度－投与後発作頻度)/投与前発作頻度を示す．

ブユニットであるGluN2B（NR2B，GluRε2）およびGluN1（NR1，GluRζ1）に対する抗体測定法を，GluN2BおよびGluN1のN末（NT）とC末（CT）の合成ペプチドを抗原としたELISAで開発し，髄液で測定した[5)~7)]．

脳炎を病因とする部分てんかん症例（PE-E）では脳炎以外の病因の部分てんかん症例（PE-NE）に比べて有意にGluN2B-NT2抗体が髄液中で高値であったが（Mann Whitney test，$p<0.0001$），症候性全般てんかん（SGE），West症候群では，脳炎を病因とする症例と脳炎以外の病因の症例で有意差が認められなかった（図5）．髄液GluN2B-CT抗体も，脳炎を病因とすると考えられる部分てんかん症例（PE-E）では脳炎以外の病因の部分てんかん症例に比べて有意に髄液中で高値であったが（$p=0.0001$），SGE，West症候群では，脳炎を病因とする症例と脳炎以外の病因の症例で有意差が認められなかった（図5）．髄液GluN1-NT抗体では，脳炎を病因とする症例と脳炎以外の病因の症例で有意差が認められなかったが，髄液GluN1-CT抗体では，脳炎を病因とする部分てんかん症例と脳炎以外の病因の症例で有意差が認められた（$p=0.0166$）（図6）．このように脳炎後の部分てんかん症例では髄液中のGluN2B抗体やGluN1抗体が有意に高値であり，病態を修飾している可能性が高い．

NMDA型GluR抗体の作用としては，①NMDA型GluRの内在化（internalization），②アポトーシス誘導作用，③NMDA型GluRのチャネル機能（long term potentiation，LTP）抑制が知られている[6)~8)]．①NMDA型GluRの内在化は非ヘルペス性急性辺縁系脳炎，抗NMDAR脳炎などのNMDA型GluR抗体で証明されていて，NMDA型GluRに対する拮抗作用をもたらすため，精神症状，記憶認知の障害などが生じると推定している（図7）．②アポトーシス誘導作用はSLE患者の髄液などで報告されていて[9)]，神経細胞死からネットワーク再構成が起こり，てんかん原性あるいは難治化等に寄与すると推測している[10)]．③LTP抑制は抗NMDAR脳炎などのNMDA型GluR抗体で証明されていて[11)]，記憶認知障害などに関与すると推定している．

5. 血液脳関門障害の関与

血液脳関門は血管内皮細胞，基底膜，アストロサイトの足突起，周囲細胞などから構成され，中枢神経系を保護している．基底膜にあるフィブロネクチンとIV型コラーゲンはmatrix metalloproteinase-9（MMP-9）の攻撃により障害され，tissue inhibitor of metalloproteinase-1（TIMP-1）により障害が防御されることが，脳炎その他の病態で知られている．我々は46例の脳炎後てんかん症例で血清中のMMP-9とTIMP-1を検討し，脳炎後てんかんでは有意にMMP-9が高値で，TIMP-1が低値であることを見出した（図8）[12)]．MMP-9上昇は0～5歳の脳炎罹患症例で著明で，脳炎罹患後も高値を維持すると推定された．TIMP-1低値は0～20歳に脳炎罹患した症例で著明で，脳炎罹患後5年くらいかけて低下し，その後低値を維持すると推定された．

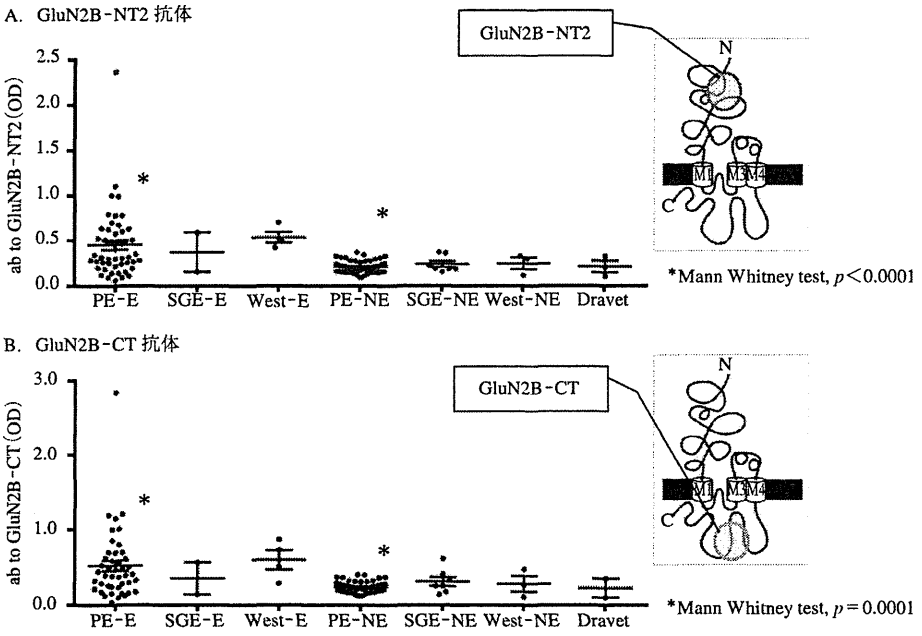

図 5 脳炎後てんかんにおける髄液 GluN2B 抗体

A：GluN2B-NT2 抗体，GluN2B（NR2B，GluRε2）の N 末ペプチドを抗原とした ELISA による抗体，B：GluN2B-CT 抗体，GluN2B（NR2B，GluRε2）の C 末ペプチドを抗原とした ELISA による抗体．
抗源位置，測定法は文献を参照されたい[5)~7)]．
PE-E: partial epilepsy after acute encephalitis, SGE-E: symptomatic generalized epilepsy after acute encephalitis, West-E: West syndrome after acute encephalitis, PE-NE: partial epilepsy by non-encephalitic causes, SGE-NE: symptomatic generalized epilepsy by non-encephalitic causes, West-NE: West syndrome by non-encephalitic causes, Dravet: Dravet syndrome.

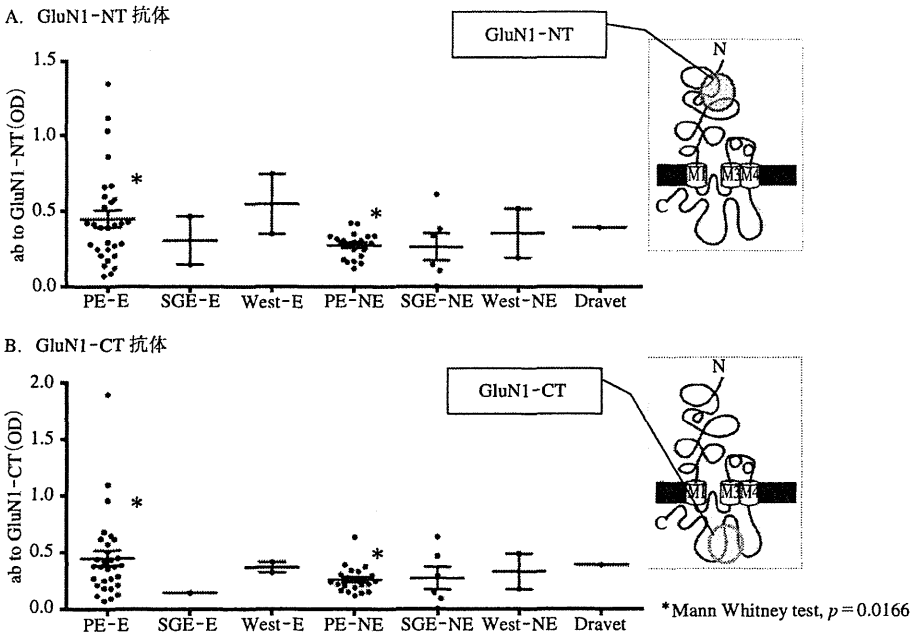

図 6 脳炎後てんかんにおける髄液 GluN1 抗体

A：GluN1-NT 抗体，GluN1（NR1，GluRζ1）の N 末ペプチドを抗原とした ELISA による抗体，B：GluN1-CT 抗体．
GluN1（NR1，GluRζ1）の C 末ペプチドを抗原とした ELISA による抗体，抗源位置，測定法は文献を参照されたい[5)~7)]．横軸の診断名は図 5 を参照．

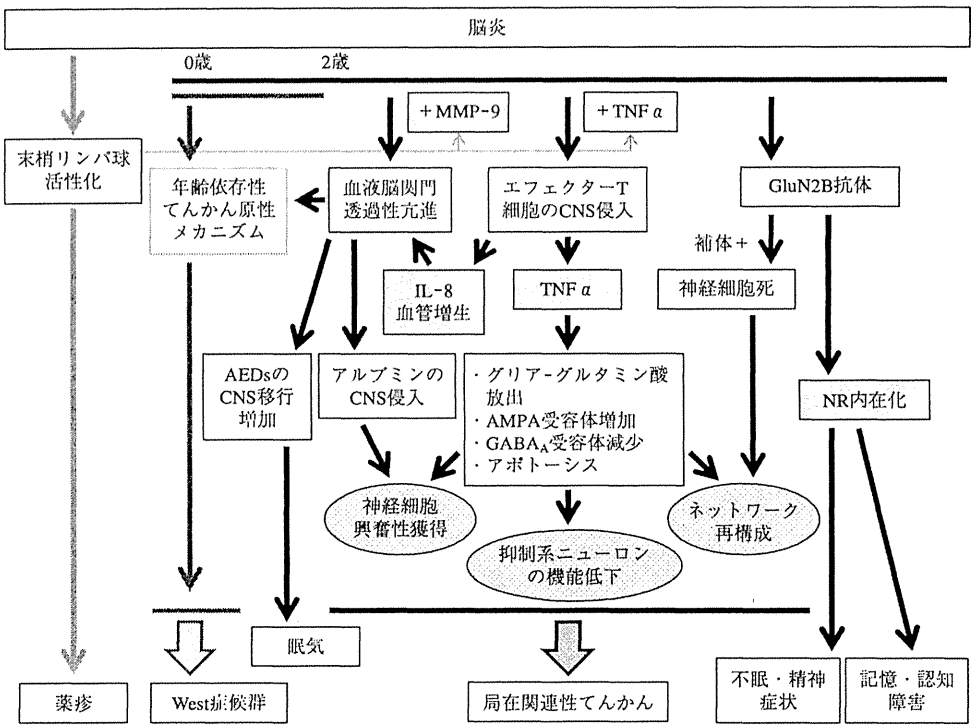

図7 脳炎後てんかんにおける免疫，生化学病態

MMP-9：matrix metalloploteinase 9，TNFα：tumor necrosis factor α，NR：N-methyl-D-aspartate（NMDA）type GluR，AMPA：alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid，GABA：γ-aminobutyric acid，CNS：central nervous system，AEDs：antiepileptic drugs.

脳炎後てんかんの血液脳関門障害は，MMP-9，TIMP-1のみならず，IL-8の幼弱血管増生作用も関与している可能性が大きい[13]．

脳炎後5年かけて血液脳関門障害が進行性に悪化することが，てんかん発作・知的障害の慢性期の悪化の一因となっていると推測している．血液脳関門障害により，髄液TNF αなどのサイトカインが増加し，神経細胞死が増え[14]，AMPA型GluR増加とGABA$_A$受容体を減少が起こり，神経興奮性を高めている可能性がある[15]．血液脳関門障害により髄液GluN2B抗体なども上昇し，LTP抑制が起こっている可能性がある．

脳炎後てんかん症例での抗てんかん薬治療では，38.8%と高頻度に眠気が出現するが，血液脳関門の障害で抗てんかん薬の中枢神経系への移行が強いためと推測している[13]．

6．高頻度の薬疹

脳炎後てんかんでは薬疹が23.9%と高頻度に出現する[16]．薬疹症例では血清RANTESが高く，薬疹出現に関与している可能性がある．脳炎後てんかんの病態には免疫応答が関与しており，アレルギー反応を含め副作用が起こりやすい状態である可能性があると思われる．ただし，急性期に薬疹が出ても，DLSTなどを参考に再使用可能な場合がある．

ま と め

脳炎後てんかんでは，自己抗体やサイトカインなどの免疫因子，MMP-9やTIMP-1などの生化学因子が病態に関与して，難治なてんかん発作，併存症の問題が起こっている可能性がある．今後さらなる検討が必要である．

第55回日本小児神経学会総会において貴重な発表の機会をお与えいただきました，泉達郎教授，高橋孝雄教授，廣瀬伸一教授に深謝申し上げます．この研究は下記の研究費の支援を受けて行われた，科研費21591342，23591238，24591537；厚生科研障害者対策総合研究；難治性疾患等克服研究；てんかん治療振興財団；平成25年国立病院機構 政策医療ネットワーク共同研究．

著者の利益相反：本論文発表内容に関連して開示すべき事項なし．

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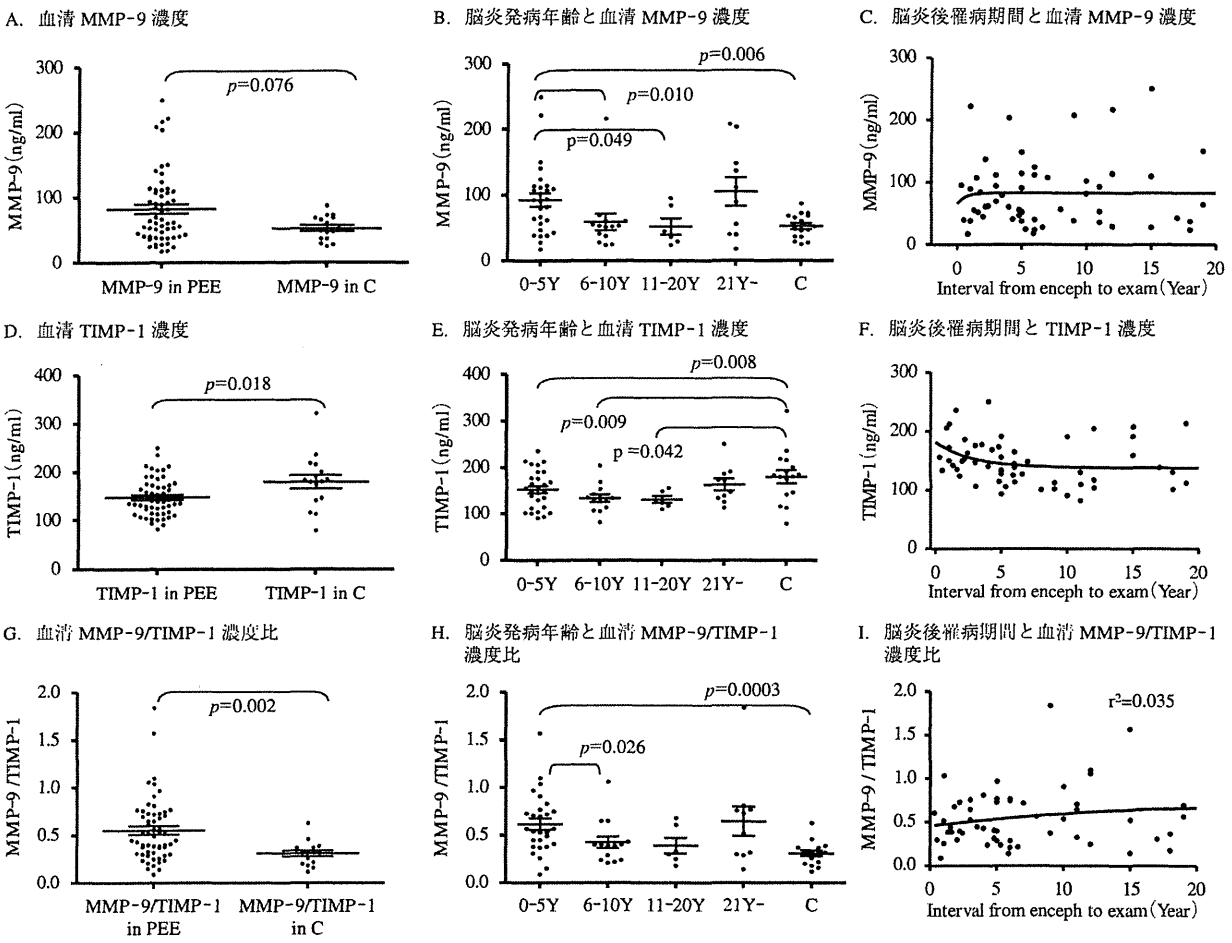

図 8 脳炎後てんかんにおける血液脳関門障害

PEE：脳炎後てんかん（postencephalitic epilepsy），C：対照（control subjects）．
MMP-9（matrix metalloploteinase-9）は activity assay kit（Amersham, Buckinghamshire, England）で測定．TIMP-1（tissue inhibitor of metalloproteinase-1）は sandwich type ELISA kit（Daiichi Fine Chemical Co., Ltd.）で測定．グラフ内の長い横棒は平均値，短い横棒は±standard error を示す．

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原著

両手指の麻痺性拘縮を呈した 抗NMDA型グルタミン酸受容体抗体陽性脳炎の一例

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仲地 耕[1)]、宮川真一[2)]、髙橋幸利[3)]

要 旨

症例は39歳の男性。2013年入院30日前より物忘れを自覚。入院20日前に全身性痙攣を初発。入院15日前より精神興奮状態となり精神科措置入院を経て当科に転院。除皮質硬直様に頸部～四肢の筋緊張が著明で、発作性に拳を握りしめたまま上肢を拳上し奇声をあげる発作が約1時間持続。血圧上昇や頻脈、高体温を伴った。脳波検査で背景脳波は全般性徐波(2Hz δ波)の持続を認め、てんかん原性異常を認めず非痙攣性てんかん重積を否定。脳MRIでは辺縁系を含めて異常所見はなかった。自己免疫介在性脳炎と臨床診断し、ステロイド/ヒト免疫グロブリン大量静注療法を施行。2クール目を施注後の入院35日目に意識障害と全身緊張発作が軽減。その時点で両手指の麻痺性拘縮が発覚し、日常生活動作が不可能な状態であった。ボツリヌス治療を手内筋などに施注し、作業療法が可能となった。本例の両手指の麻痺性拘縮は、脳幹網様体脊髄路から脊髄前角へのシナプスにおけるNMDA型グルタミン酸受容体の自己免疫介在性機序による障害で、痙縮に類似した上位運動ニューロン徴候を呈したものと考察した。

Key words: 抗NMDA型グルタミン酸受容体抗体陽性脳炎、抗NMDA型グルタミン酸受容体抗体、麻痺性拘縮、網様体脊髄路、ボツリヌス治療、ヒト免疫グロブリン大量静注療法。

はじめに

近年、辺縁系脳炎をはじめとして、精神神経症状を呈する患者の血清・脊髄液に神経細胞表面に結合し病態に関与する疾患特異性の高い自己抗体が報告されるようになり、自己免疫介在性脳炎と総括されている[1)]。なかでも、2007年にDalmauらがNMDA受容体(anti-N-methy-D asparate receptor: NMDAR)に対する自己抗体の存在を、卵巣奇形腫を伴う辺縁系脳炎例の血清と脊髄液中で明らかにして以降、抗NMDA受容体脳炎と表記されるようになった。本症は特異的な症状の広がりと経過を示し、辺縁系脳炎の括りには収まらない。

我々は全身性緊張発作が約5週間持続した後に、両手指の麻痺性拘縮を生じた一例を経験した。NMDA型グルタミン酸受容体(NMDA-GluR)の脳幹～脊髄下行路における興奮性シナプス伝達における機能を文献的に検討し本例の麻痺性拘縮の機序について考察した。