Supporting Information

Ohmi et al. 10.1073/pnas.0912336106

SI Text

Mice. Complement component 3-defective mice (B6.129S4-*C3*^{tm1Crr}/J) were obtained from The Jackson Laboratory. GM2/GD2 synthase and GD3 synthase genes KO mice were mated with C3 KO mice, and genotypes of the offspring were screened for the three genes. Genotypes in C3 KO mice were screened as described (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol& protocol_id=407).

Extraction of Glycolipids and TLC. Glycolipid extraction and TLC were performed as described in ref. 6.

DNA Microarray. The cerebella and spinal cords isolated from 28- and 48-week-old mice ($n = 3$) were homogenized in TRIzol, and total RNA was extracted from tissues following the manufacturer's protocol (Invitrogen). Comparison of the gene expression profiles was performed using pooled RNAs from three mice. DNA gene chip analysis was performed depending on custom analysis services (KURABO).

Hematoxylin-Eosin (H&E) Staining. WT and DKO mice were perfused with PBS (PBS) and then 10% formalin neutral buffer solution (Wako), and were postfixed with 10% formalin neutral buffer solution. The cerebellum isolated from mouse was cut in ≈3-mm sections and embedded in paraffin using Tissue Tek VIP5 junior (Sakura) after dehydration and paraffin penetration. Blocked cerebellum was cut into 3-μm sections and used for H&E staining. H&E-stained slices of the cerebellum were observed by light microscopy.

Antibodies. Antibodies used for western immunoblotting were as follows: anti-mouse C1q mAb (rat IgG1) (Hycult Biotechnology), anti-β-actin mAb (Sigma), goat anti-mouse DAF (Santa Cruz Biotechnology), anti-rat flotillin-1 mAb (mouse IgG1) (BD Biosciences), anti-mouse Thy1.2 mAb (rat IgG1) (AbD Serotec), anti-mouse NCAM mAb (rat IgG2a) (Abcam), and rabbit anti-human Caveolin-1 (Santa Cruz Biotechnology). Chemiluminescence detection was performed using HRP-conjugated rabbit anti-rat IgG (H+L) (ZYMED Laboratories), sheep anti-mouse IgG (Amersham Biosciences), horse anti-goat IgG (H+L) (Vector Laboratories). Antibodies used for immunohistochemistry were as follows: anti-mouse C1q mAb (rat IgG1) (Hycult Biotechnology), anti-mouse C3b/iC3b/C3c mAb (rat IgG1) (Hycult Biotechnology), anti-mouse DAF mAb (mouse IgG) (a kind present from Dr. Mizuno at Nagoya University), anti-human CD59 mAb (mouse IgG2b) (Bio Vendor), rabbit anti-human flotillin-1 (Santa Cruz). Immunofluorescence detection reagents were purchased from Invitrogen; that is, Alexa Fluor 488-goat anti-mouse IgG2b, Alexa Fluor 488-goat anti-rat IgG, Alexa Fluor 555-goat anti-mouse IgG1, Alexa Fluor 488-goat anti-rabbit IgG, or Alexa Fluor 546-goat anti-mouse IgG.

Fractionation of Extracts with Sucrose Density Gradient Ultracentrifugation. All steps were performed on ice. After perfusion with PBS, tissues were isolated and minced in PBS containing 1 mM PMSF followed by nitrogen cavitation. Nitrogen-cavitated samples were centrifuged at 1,000 rpm for 10 min at 4 °C. The supernatant fractions were centrifuged at 100,000 × g for 1 h at 4 °C (Beckman Coulter, Optima MAX-E), and the obtained pelleted membrane fractions were resuspended in 1.2 mL MNE buffer (25 mM Mes, pH 6.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, and 0.01–0.02 TIU/mL aprotinin) containing 1% Triton X-100, and treated with 10 strokes of a Dounce homogenizer (Iuchi). Resuspended samples (1 mL) were then added to an equal volume of 80% sucrose in MNE buffer in an ultracentrifuge tube and overlaid with 3 mL discontinuous sucrose gradient, consisting of 30% sucrose in MNE buffer (2 mL) and 5% sucrose in MNE buffer (1 mL). Ultracentrifugation was performed at 100,000 × g for 16 h at 4 °C in an Optima MAX-E (Beckman Coulter). Fractions containing 0.5 mL each were obtained from the top to the bottom of the tube.

ELISA. After perfusion with PBS, tissues were isolated, homogenized and sonicated in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 1 mM $NaVO_4$, 1% Triton X-100, 1 mM PMSF, and 0.01–0.02 TIU/mL aprotinin). The lysates were centrifuged at 8,000 × g for 60 min at 4 °C, then the supernatants were centrifuged at 18,000 × g for 90 min at 4 °C. Clarified lysates were used for ELISA. Levels of IL-1α, IL-1β, and TNFα proteins were determined using assay kits for mouse IL-1α/IL-1F1, mouse IL-1β/IL-1F2 ELISA, or mouse TNFα/TNFF1A (R&D Systems). The assay was performed according to the manufacturer's instructions.

Genes of Complement Components Were Up-Regulated in the Cerebellum of DKO Mice. To precisely determine gene expression profiles, real time RT-PCR was performed with mRNAs from individual mice. Consequently, expression levels of *C3aR* were up-regulated in DKO mice compared to WT at all ages examined, except in 48-week-old females (Fig. S1).

Complement Proteins Increased in Young DKO Mice. We performed immunoblotting to investigate the protein level of C1q which is essential in the complement system and up-regulated in the DKO mice as described above. C1q protein levels in the cerebellum of 15-week-old DKO mice were increased 3-fold compared with WT (Fig. S2*A*), although they were almost equivalent at 30 weeks (Fig. S2*B*), and decreased at 60 weeks in DKO mice (Fig. S2*C*) even though C1q mRNA levels were up-regulated with aging. To determine whether C1q was activated in DKO mice, deposits of C1q were analyzed with immunohistostaining. At both 15 (Fig. S2 *D* and *F*) and 30 weeks (Fig. S2 *E* and *G*) deposits of C1q increased in the cerebellum of DKO mice, suggesting progressive activation of C1q at 15 weeks and thereafter.

CD59 Was Dispersed from GEM/Rafts in the Cerebellum of DKO Mice. CD59 also stained distinctly when comparing DKO and the WT mice (Fig. S3).

Alleviation of the Inflammatory Reaction in the DKO Mice by Genetic Disruption of Complement C3. To investigate whether the inflammatory reaction in DKO mice associated with complement systems, we generated triple KO (TKO) mice by mating DKO with C3 KO mice. Deposits of C3b/iC3b/C3c (active forms of C3) as well as C4 were examined by immunohistostaining. TKO mice showed no deposits (Fig. S4), indicating that complement activation was completely suppressed in TKO mice.

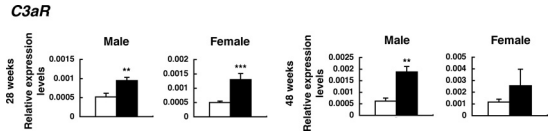

Fig. S1. mRNA levels of *C3aR* were up-regulated in the cerebellum of DKO mice. Expression of the *C3aR* mRNA levels were analyzed by real time RT-PCR and presented after correction with *GAPDH* gene. The number of mice examined was: 28-week-old male WT $n = 3$, DKO $n = 3$; 28-week-old female WT $n = 3$, DKO $n = 6$; 48-week-old male WT $n = 3$, DKO $n = 3$; 48-week-old female WT $n = 3$, DKO $n = 5$; data are presented as mean $\pm$ SD. **, $P < 0.01$; ***, $P < 0.001$.

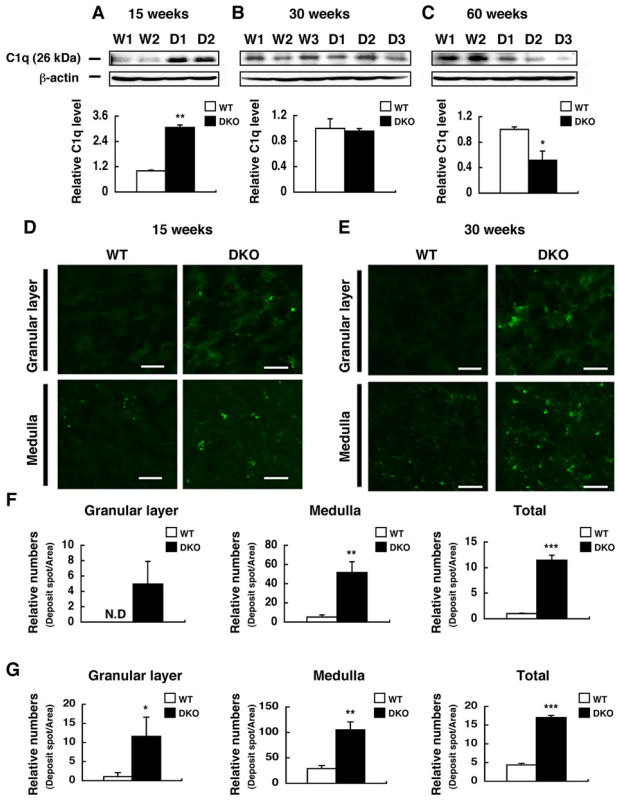

Fig. S2. Complement C1q levels in the cerebellum as analyzed by immunoblotting and immunohistostaining. (*A–C*) Western immunoblotting of C1q in the cerebellum was performed using total lysates from 15-, 30-, and 60-week-old WT and DKO mice. The intensity of the bands was corrected with that of β-actin and presented as mean $\pm$ SD. The numbers of mice examined was: 15-week-old WT $n = 2$, DKO $= 2$; 30-week-old WT $n = 3$, DKO $n = 3$; 60-week-old WT $n = 2$, DKO $n = 3$. Each mouse is designated as W or D plus number. (*D–G*) Deposits of C1q in the cerebella of 15- (*D* and *F*) and 30-week-old (*E* and *G*) WT and DKO mice were analyzed with anti-C1q mAb and Alexa Flour 488-conjugated anti-rat IgG. Data were presented as number of detected spots per 800 $\times$ 800-μm space of the individual layers of cerebellum section (*F* and *G*). The thickness of sections was 7 μm. [Scale bar, 20 μm (*D* and *E*).] The number of mice examined was: 30-week-old WT $n = 3$, DKO $n = 3$; data are presented as mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

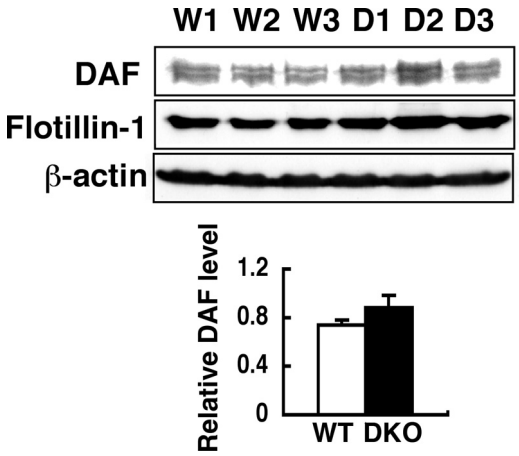

Fig. S3. Total protein levels of DAF were not different in the cerebellum of WT and DKO mice. Western immunoblotting of DAF and flotillin-1 was performed using whole lysates of the cerebellum to examine total protein levels. DAF bands were corrected by those of β-actin. The number of mice examined was: 30-week-old WT $n = 3$, DKO $n = 3$; data are presented as mean $\pm$ SD. No significant differences were found.

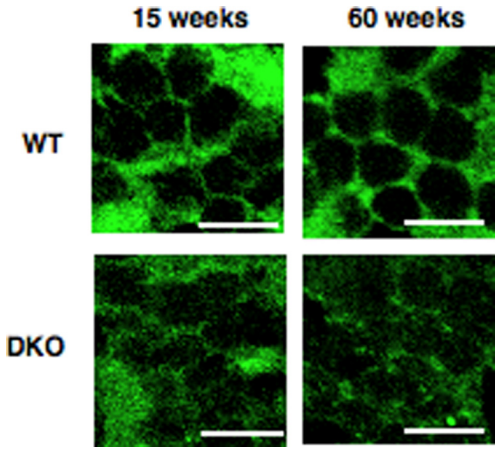

Fig. S4. The CD59 distribution pattern was altered in the cerebellum of DKO mice. Confocal microscopic imaging of granular cells of 15- and 60-week-old cerebellum labeled with an anti-CD59 mAb, demonstrating that CD59 was stained on both cell membranes and cytoplasm of the DKO mice, while WT mice showed staining only on cell membranes. (Scale bar, 10 μm.)

Fig. S5. Alleviation of the complement activation and inflammation in the DKO mice by disruption of complement C3 gene. Deposits of C3b/iC3b/C3c in the cerebella of 15-week-old WT, DKO, TKO, and C3 KO mice were analyzed with anti-C3b/iC3b/C3c mAb in combination with Alexa Flour 488-conjugated anti-rat IgG. Fluorescence intensity (mean pixel $\times$ total area) of complement staining was measured by digital image analysis. Number of section area examined was: 15-week-old WT $n = 6$, DKO $n = 6$ TKO $n = 6$, C3 KO $n = 6$; data are presented as mean $\pm$ SD. The thickness of sections was 7 μm. (Scale bar, 20 μm.) Data are presented as mean $\pm$ SD. (Scale bar, 50 μm.) *, $P < 0.05$; ***, $P < 0.001$.

Movie S1. Behavior of a DKO mouse (*Upper*) and a WT mouse (*Lower*).

Movie S1 (MP4)

Table S1. Expression levels of complement components using DNA microarray

Tissue and weeks	Gene	WT (expression)*	DKO (expression)*	Ratio
Cerebellum (28 weeks)	*C1qα*	5.27	9.14	1.74
	C1qγ	5.42	8.21	1.51
	C4	0.87	3.77	4.33
	C3aR	0.18	0.37	2.08
Cerebellum (48 weeks)	*C1qα*	4.27	12.76	2.99
	C1qγ	5.19	10.84	2.09
	C3	0.07	0.32	4.53
	C4	1.37	5.00	3.66

*Expression levels of genes in WT and DKO mice were quantified based on normalized signal intensities (see *Experimental Procedures* in the main text) obtained from DNA microarray.

Table S2. Sequences of primers used for real time RT-PCR

Gene	NCBI (accession no.)	Sense	Antisense	Product size (bp)
C1qα	NM_007572	acaaggtcctcaccaaccag	aagatgctgtcggcttcagt	307
C1qβ	NM_007574	tggcactccagggattaaag	atagttctcgttcgcgttgg	301
C1qγ	NM_009777	agtgccgggcctctactact	cgggaaacagtaggaaacca	251
C2	NM_013484	atccgtcacaccatcatcct	ttctttccagtccacatcca	168
C3	NM_009778	aactgctggcctctggagta	gcatgattcctcgaggttgt	212
*C3**	NM_009778	aactgctggcctctggagta	ctgtgttgttcacgcagctt	213
C4	NM_009780	ttaagacccacgggctacac	tggcatgtggtgttcttcttcat	169
C5	NM_010406	gctcttgtggaaggagtgga	aagcctggtgtcagaaatgc	228
C6	NM_016704	ctcaaccacctctcccagaa	ccagcaacaacaaagcctct	286
C7	XM_356827	cctcccagagatgatggaaa	ctggcaacaggatctccaat	257
C8α	AK090245	actgggtagagggcttccat	tggtctgtctgcttgtccag	212
C8β	AK050313	cttctgtcaggttggcttcc	cagaccagtcagaccagcaa	310
C8γ	AK005962	cttctgtcaggttggcttcc	tggtggttgctgagactgac	156
C9	NM_013485	aaggtttcgctcaagaagca	tccacagtcgttgtcaccat	199
C3aR	NM_007577	tgacaggtcagctccttcct	cattaggaggctttccacca	266
C5aR	NM_007577	caagacgctcaaagtggtga	tatgatgctggggagagacc	244
IL-1α	NM_010554	cgaagctctccgtacattcc	gaatccaggggaaacactga	263
IL-1β	NM_008361	gcccatcctctgtgactcat	aggccacaggtattttgtcg	230
TNFα	NM_013693	ccacatctccctccagaaaa	agggtctgggccatagaact	259
mGAPDH	NM_008084	ggtgctgagtatgtcgtgga	ccttccacaatgccaaagtt	248

Primers used for real time RT-PCR were designed according to Primer 3 Input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).
*Primers used for TKO mice.