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Signaling Pathways in the Activation of Mast Cells Cocultured with Astrocytes and Colocalization of Both Cells in Experimental Allergic Encephalomyelitis

Dae Yong Kim,* Dooil Jeoung, † and Jai Youl Ro*

Mast cells in the CNS participate in the pathophysiology of chronic neurodegenerative inflammatory diseases. This study aimed to investigate the signaling pathway of mast cells activated in an environment cocultured with astrocytes and to explore the role of their colocalization in brain of experimental allergic encephalomyelitis. Human mast cell line-1 cells and human U87 glioblastoma cell lines (U87) or mouse bone marrow-derived mast cells and mouse cerebral cortices-derived astrocytes were cocultured. Intracellular Ca2+ was measured by confocal microscopy; histamine by fluorometric analyzer; leukotrienes by ELISA; small GTPases, protein kinase C, MAPK, c-kit, CD40, and CD40L by Western blot; NF-κB and AP-1 by EMSA; cytokines by RT-PCR; and colocalization of mast cells and astrocytes in brain by immunohistochemistry. Mast cells cocultured with astrocytes showed time-dependent increases in intracellular Ca2+ levels, release of histamine and leukotrienes, and cytokine production. Mast cells or astrocytes showed enhanced surface expression of CD40L and CD40, respectively, during coculture. Mast cells cocultured with astrocytes induced small GTPases (Rac1/2, cdc42), protein kinase C, MAPK, NF-κB, and AP-1 activities. These changes were blocked by anti-CD40 Ab pretreatment or CD40 small interfering RNA. Mast cells increased in the thalamus of experimental allergic encephalomyelitis model, particularly colocalized with astrocytes in the thalamic border region of the habenula. In conclusion, the data suggest that activation of mast cells cocultured with astrocytes induces release of mediators by small GTPases/Ca2+ influx through CD40–CD40L interactions to participate in the pathophysiology of chronic neurodegenerative inflammatory diseases, such as multiple sclerosis. The Journal of Immunology, 2010, 185: 273–283.

Abbreviations used in this paper: + anti-CD40, anti-CD40 Ab pretreatment; 2-APB, 2-aminoethoxydiphenyl borate; 8-oxo-dG, 8-hydroxydeoxyguanosine; a, negative control; b, competition assay; BBB, blood-brain barrier; BM, BMMCs cultured alone; BMMC, bone marrow-derived mast cell; [Ca2+]i, intracellular Ca2+; CD40 siRNA, HMC-1 cells cocultured with transfected U87 cells; EAE, experimental allergic encephalomyelitis; GFAP, glial fibrillary acidic protein; HMC, HMC-1 cells cultured alone; HMC-1, human mast cell line-1; LT, leukotrienes; MIG, monokine induced by IFN-γ; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBST, PBS containing 0.1% Tween 20; PKC, protein kinase C; siRNA, small interfering RNA.

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can produce cytokines or chemokines that cause mast cell degranulation (16). We therefore hypothesized that mast cells may interact with astrocytes via interactions of cell surface molecules in the brain. To pursue this hypothesis, we investigated the signaling pathways activated in mast cells during coculture with astrocytes. We demonstrated that mast cells were activated with astrocytes through cross-talk of CD40–CD40L.

Materials and Methods

Cell line culture

Human mast cell line-1 (HMC-1) cells were provided by J. H. Butterfield (Minneapolis, MN). Cells were grown in IMDM (Life Technologies, Carlsbad, CA) containing 10−5 M monothioglycolater, 100 U/ml penicillin/streptomycin (Life Technologies), and 10% heat-inactivated FBS at 37°C in a 5% CO2 atmosphere. These culture conditions were designated as control medium.

U87 glioblastoma cell lines were obtained from Korea Cell Line Bank (Seoul, Korea); grown in DMEM (Life Technologies) formulated with 4.5 mM t-glutamate, 4.5 mg/ml t-glucose, and 110 µg/ml sodium pyruvate; and supplemented with 10% FBS and 100 U/ml penicillin/streptomycin.

Preparation of bone marrow-derived mast cells and primary astrocytes

Bone marrow cells were flushed from femurs and tibias of BALB/c mice (female, 8 wk old). RBCs were lysed using what 0.1 M NH4Cl, and the remaining cells were washed, resuspended, and cultured in RPMI 1640 supplemented with 10% FBS and 50% WEHI-3B conditioned media, which contains IL-3, for 5 wk. Bone marrow-derived mast cells (BMMCs; ∼105 cells) were collected onto object glasses by cyto centrifuge (400 × g, 3 min). BMMCs were fixed in methanol for 2–3 min and then stained with May-Gräwald solution for 15 min, followed by Giemsa solution for 10 min and by washing steps in H2O2, confirmed under microscope (21).

Primary astrocytes were prepared from the cerebral cortices of 1- to 3-d-old BALB/c mice. In brief, animals were sacrificed by decapitation, meninges were removed, and cortices were minced and gently dissociated in HBSS. Cells were supplemented with DMEM containing 5% FBS, plated onto 75-cm2 culture flask (5 × 105 cells/dish), and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO2. After 14 d of culture, floating microglia was removed by shaking the flask vigorously. As reported previously (22), >95% of cells were stained for astrocyte-specific glial fibrillary acidic protein (GFAP, Sigma-Aldrich, St. Louis, MO).

Coculture of mast cells and astrocytes

U97 cells or primary astrocytes (1 × 106 cells) were grown in 75-cm2 flasks until confluent, and then HMC-1 cells or BMMCs (3 × 105 cells) were added to each astrocyte flask, and the cells were cocultured for up to 24 h. In vivo, brain astrocytes outnumber mast cells, and we chose a 3:1 ratio of mast cells and astrocytes to activate mast cells and produce sufficient numbers for testing. Supernatants after coculture were collected and kept at −70°C until analysis for mediators (histamine, leukotrienes [LTs], and cytokine array). After coculture, mast cells were separated from astrocytes attached to the flask by gentle shaking. Astrocytes were separated from flasks using trypsin treatment and harvest by centrifugation. The optimal concentration (300 ng/ml) and time (1 h) for anti-CD40 Ab treatment of U87 cells were obtained in preliminary experiments (Supplemental Fig. 1A, 1B).

For inhibition experiments, U87 cells or primary astrocytes (1 × 106 cells) were incubated for 1 h, and protein kinase C (PKC) inhibitors (5 µM staurosporine and Go6976), MAPK inhibitors (50 µM PD908059 for ERK, 10 µM SP600125 for JNK, and 10 µM SB203580 for p38), or 8-hydroxydeoxyguanosine (8-oxo-dG; 300 µg/ml) were pretreated 10 min before initiating coculture.

Measurement of intracellular Ca2+ level

HMC-1 cells or BMMCs (3 × 105 cells) cocultured with astrocytes (1 × 106 cells) were incubated for 30 min after adding fluo-3-acetoxymethyl ester (5 µM) and placed on a glass slide treated with poly-L-lysine. The intracellular Ca2+ ([Ca2+]i) levels in cocultured mast cells were quantified with fluorescence intensity using a LSM 510 laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) (23).

CD40 small interfering RNA transfection

CD40 small interfering RNA (siRNA)-expressing vectors were generated using the Silencer Express Kit (Ambion, Austin, TX). Sense (5′-ACA CTA CAC AAA TGT TCT AGG CTG AGA ACC GGT GTT TCG TCC TTT CCA CAA G3′-3′) and antisense (5′-CCG CAG ACC GTT TTC TCT CAA AAA ATC CTC AGC CCA GTG GAA CAC TAC ACA AAT G3′-3′) hairpin siRNA template oligonucleotides, specific to CD40 mRNA, were used (24).

Transfection was performed according to the manufacturer’s method. Briefly, 1 µg vector expressing CD40 siRNA or control siRNA was incubated with 50 µl serum-free media for 5 min (solution A), and 2 µl lipofectamine 2000 (Life Technologies) incubated with serum-free media for 5 min (solution B). Solution A was mixed with solution B, and incubated for 20 min. After incubation, U87 cells were added to the mixture (Supplemental Fig. 7). Transfected U87 cells were co-cultured with HMC-1 cells, and then the [Ca2+]i level, Rho families, PKC isoforms, and MAPKs were measured using a GST effector pull-down assay and Western blot, respectively.

Histamine assay

Histamine in supernatants obtained from cocultured cells was quantified using an automated fluorometric analyzer (Astorina analyzer series 300, Clarkan, OR) (23). The detection limit of the histamine assay was ∼5 ng/ml, and the amount of histamine released was expressed as a percentage of the total histamine content in unstimulated mast cells.

LT immunoassay

The LT content of supernatants obtained from cocultured cells was determined using an enzyme immunoassay kit according to the manufacturer’s instructions. Briefly, 50 µl samples were incubated with anti-LT antiserum (diluted 1/120) and acetylcholinesterase-linked LTs (diluted 1/120) in wells that had been coated with mouse mAbs for 18 h at room temperature. After rinsing with washing buffer, color was developed using Ellman’s reagent, and the plates were read at 412 nm with a spectrophotometer. The concentrations of LTs were then calculated using standard curves generated with specific LT standards. The results are expressed in ng/ml (1 × 105 cells). The LT concentration was calculated using analysis tools on the Cayman Chemical (Ann Arbor, MI) Web site (www.caymanchem.com/app/template/analysis%2CEIA.vm/a/z). Cytokines secreted in media after coculture using cytokine Ab array

The membranes included in a human protein cytokine array kit (RayBiotech, Norcross, GA) were blocked with a blocking buffer, and then 1 ml cocultured media was added and incubated at room temperature for 2 h. After incubation, each membrane was thoroughly washed with the provided washing buffers and incubated for 1–2 h at room temperature with biotin-conjugated anti-cytokine Ab, followed by the incubation of streptavidin–HRP conjugate. After several washes, cytokine–Ab complexes were detected by chemiluminescence using reagents and procedures provided by RayBiotech. Levels of individual cytokines were assessed semiquantitatively using a LAS-3000 (Fujifilm, Tokyo, Japan).

GST effector pull-down assay

Small GTPase protein activities were assays, as previously described (25), using EZ-Detect protein activation kits (Upstate Biotechnology, Lake Placid, NY). Cocultured HMC-1 cells or BMMCs (3 × 105 cells) were suspended in 0.5 ml lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 1 mM DTT, 5% glycerol, 1 mM PMSEF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin) for 30 min on ice, and supernatants were obtained by centrifugation (13,000 × g for 20 min). According to the manufacturer’s protocol, the active form of small GTPase proteins was obtained from the supernatants by affinity precipitation using Pak-1 protein binding domain, which was fused to GST, and visualized by Western blot analysis with anti-rabbit RasC12, cdc42 (1:1000).

Western blot

Cocultured HMC-1 cells or BMMCs (3 × 105 cells/50 ml) were homogenized in lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSEF, 2.0 µg/ml aprotinin, and 2.0 µg/ml leupeptin), and allowed to swell on ice for 30 min. Cell lysates (µg) were subjected to 8–10% SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, United Kingdom). Membranes were washed with PBS containing 0.1% Tween 20 (PBST) and then blocked for 1 h in PBST containing 5% skim milk. After washing the membranes with PBST, they were treated with Abs against PKC isoforms, ERK, JNK, p38, c-kit (Santa Cruz Biotechnology, Santa Cruz, CA), CD40, and CD40L (NeoMarkers, Fremont, CA), and p-PKC by guest on September 15, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/
isomers, p-ERK, p-JNK, and p-p38 (Cell Signaling Technology, Beverly, MA) diluted with PBST (1/1,000), and incubated for 60 min at room temperature. Membranes were washed with PBST, and treated with HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG (diluted to 1/5,000; Zymed Laboratory, San Francisco, CA) in PBST for 60 min. After washing, the protein bands were visualized using electrogenerated chemiluminescent (ECL) solution (Amer sham Biosciences) (25).

Cytokine levels secreted in media after coculture

Amounts of cytokines and chemokines (IL-1β, IL-4, IL-6, TNF-α, IFN-γ, MCP-1, monokine induced by IFN-γ [MIG], and RANTES) secreted into coculture media were determined using an ELISA kit. Coculture media were centrifuged at 400 × g for 5 min, and then supernatants were collected. Standard curves were generated using different concentrations of the recombinant cytokines. The limit of detection of this method was >7.8 pg/ml.

RT-PCR

Total cellular RNA was isolated from cocultured HMC-1 cells or BMMCs (3 × 10^6 cells) using TRI-Reagent (Molecular Research Center, Cincinnati, OH). RT-PCR was performed in a final volume of 50 μl using an amplifi er one-step RT-PCR kit (GenDEPOT, Barker, TX) in an automated thermal cycler (BIOER XP cycler; BIOER Technology, Hangzhou, China). PCR assays were performed for 35 cycles. Each cycle consisted of the following steps: denaturation at 94˚C for 30 s, annealing at 56˚C for 45 s, and extension at 72˚C for 1 min. PCR products were analyzed using 1% agarose gel containing ethidium bromide (25).

The primer sequences used were as follows: human IL-6 sense, 5'-GCC TTC TTT TGG GAA GTG GAT GAG TGT TTG TC-3' and antisense, 5'-GGAA TGA TGA TCT GGC TTT-3'; and mouse IL-6 sense, 5'-TGG AGT CAC AGA AGG AGT GAG TGT TTG TC-3' and antisense, 5'-TGG AGT GCA GGG ATG ATG TTC TG-3'.

EMSA

Nuclear extracts were prepared from cocultured HMC-1 cells or BMMCs (3 × 10^6 cells). Cells were washed twice with ice-cold PBS, and resuspended in 1 ml ice-cold buffer A (10 mM HEPES/KOH [pH 7.9], 10 mM KC1, 1.5 mM MgCl2, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After incubation on ice for 15 min, the cells were lysed by adding Nonidet P-40 (10 μl 10% Nonidet P-40, to a final concentration of 0.625%, v/v) and immediately vortexed for 10 s. Nuclei were harvested by centrifugation at 20,000 × g for 5 min, washed twice in ice-cold buffer C (20 mM HEPES/KOH [pH 7.9], 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After incubation at 4˚C for 20 min on a shaking platform, the nuclei were clarified by centrifugation at 15,000 × g for 10 min. The supernatant (nuclear extract) was then transferred to a new tube, and quantified using Bradford's method. Nuclear extract was stored at −70˚C until required (26).

Ten microliters of a mixture of NF-κB (5'-AGT TGA GGA TGT TCC CCC AGG C-3', 5'-TCA ACT CCC CTG AAA GGA TCC G-5') or AP-1 (5'-GGCC TGG TTG AGT AGT CCG GAA-3', 5'-GCG AAC TAC TCA GTC GGC CCT-5') oligonucleotide (1.75 pmol/μl), T4 polynucleotide kinase 10X buffer, [α-32P]-P ATP (10 μCi; 3000 Ci/mmol), nuclear-free water, and T4 polynucleotide kinase (∼5–10 U/μl) were incubated at 37˚C for 30 min. The reaction was stopped by adding 1 μl EDTA (0.5 M). After adding 89 μl Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), unincorporated nucleotides were removed from the DNA probe by chromatography through a G-25 spin column. The nuclear extract and gel shift binding 5X buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl [pH 7.5], and 0.25 mg/ml poly[dI-dC]) were incubated at room temperature for 10 min, and then 20–30 fmol (32P)-labeled NF-κB or AP-1 oligonucleotide was added and incubated at room temperature for 20 min. After stopping the reaction, 1 μl 10X gel-loading buffer was added to each reaction. Reaction mixtures were electrophoresed on 6% polyacylamide gels, and gels were analyzed using FLA-2000 (Fujiﬁ lm).

Induction of EAE

Female mice (C57BL/6; 8 wk) were purchased from Samtako BioKorea (Osan, Korea) and maintained in specific pathogen-free condition before sacrifice. All mice were housed in accordance with guidelines from the Association for Assessment and Accreditation of Laboratory Animal Care, and all protocols were approved by the Institutional Review Board and conducted in the Laboratory Animal Research Center of Sungkyunkwan University.

For active induction of EAE, mice (eight mice/group) received s.c. injection of 150 μg myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (Sigma-Aldrich). Animals injected with CFA alone were used as controls. The next day, each animal received an i.p. injection of 200 ng pertussis toxin (Life Technologies) in 200 μl PBS. The mice were weighed and scored daily in a blinded fashion by two examiners, according to the following scale: score 0, no disease; score 1, loss of weight and tail weakness; score 2, weakness in hind limb; score 3, complete hind limb paralysis; score 4, hind limb paralysis with fore limb weakness or paralysis; and score 5, moribund or deceased (27).

Thirty-two days after starting injection, the EAE score was ≥3.1, and brains were isolated. Brain tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut at 3 μm. Brain sections were then stained with May–Grünwald-Giemsa for mast cells (21). Brain sections were deparaff inized with xylene and washed in ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. Slides were then blocked with 1% BSA in PBS for 1 h. For immunohistochemistry, a polyclonal primary Ab to c-kit, GFAP, CD40L (Santa Cruz Biotechnology; 1/50 dilution), or CD40 (Neomarkers; 1/25 dilution) was applied and incubated at 4˚C for 24 h. After washing in PBS, slides were treated with biotinylated secondary Ab for 10 min, streptavidin-HRP for 10 min, and chromogen substrate (Dako-Cytomation, Carpinteria, CA) for 5–10 min. Thorough 1-min wash steps were performed between each stage. Slides were counterstained with hematoxylin (Sigma-Aldrich) and finally mounted using aqueous mounting medium (Dako-Cytomation). For immunofluorescence, a polyclonal primary Ab to c-kit or GFAP was then applied and incubated at 4˚C for 24 h. After washing in PBS, slides were treated with the corresponding FITC- or Texas-Red–conjugated anti-IgG for 1 h at room temperature. After washing, the slides were mounted using aqueous mounting medium, and examined using a confocal microscope (LSM 5 EXCITER; Carl Zeiss) (28).

Statistical analysis

Experimental data are shown as means ± SEM. An unpaired Student’s t test was used to analyze the results for statistical significance when only two conditions were compared. Values of p below 0.05, 0.01, or 0.001 were considered significant.
FIGURE 1. Activation of mast cells cocultured with astrocytes. HMC-1 cells or BMMCs (3 × 10^6 cells) were cocultured with U87 cells or primary brain astrocytes (1 × 10^6 cells), respectively, for the indicated periods. The anti-CD40 Ab (300 ng/ml) was pretreated in astrocytes 1 h before coculture, and CD40 siRNA was performed, as described in Materials and Methods. Fluo-3-acetoxymethyl ester (5 μM) was added to cocultured HMC-1 cells or BMMCs and incubated for 30 min. The [Ca^{2+}]_i level in cocultured HMC-1 cells or in BMMCs was analyzed by confocal microscopy. Histamine and LTs released from both cocultured mast cells were determined using an automated fluorometric analysis and ELISA, respectively. CD40 and CD40L molecules were determined by Western blot. 

A, Intensity of fluorescence in confocal microscopy. 

B and C, Time course of [Ca^{2+}]_i in cocultured HMC-1 cells or BMMCs. 

D, Expression of CD40 or CD40L in cocultured HMC-1 cells and U87 cells. 

E and F, Histamine and LT release. 

G and H, Histamine and LT release after anti-CD40 Ab pretreatment. The data are representative of four independent experiments (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001 versus HMC-1 cells or BMMC culture alone; ++p < 0.01; +++p < 0.001 versus cocultured HMC-1 cells or cocultured BMMCs or anti-CD40 Ab pretreatment; ##p < 0.01; ###p < 0.001 versus cocultured HMC-1 cells. ♦, Numbers below bands are the ratio of band density of each group (CD40 or CD40L) versus those of control and tubulin protein. + anti-CD40, anti-CD40 Ab pretreatment; BMMCs, BMMC culture alone; CD40 siRNA, HMC-1 cells cocultured with transfected U87 cells; coculture, HMC-1 cells or BMMCs cocultured with each astrocyte; HMC-1, HMC-1 cell culture alone.
Responses. Mast cells express CD40L, a potent inducer of astrocyte activation (29), and astrocytes functionally express CD40. Cocultures of HMC-1 cells showed higher levels of CD40L and similar levels of CD40, as assessed via Western blot. Coculture increased CD40 expression in astrocytes (U-87 cells), but CD40L was not detected (Fig. 1D).

Therefore, we examined whether 300 ng/ml anti-CD40 Ab pretreatment for 1 h (optimal dose and time were 300 ng/ml and 1 h, respectively) (Supplemental Fig. 1A, 1B) inhibited [Ca2+]i levels in cocultured HMC-1 cells. Anti-CD40 Ab pretreatment time dependently decreased [Ca2+]i levels in cocultured HMC-1 cells (Fig. 1B) or BMMCs (Fig. 1C), but did not completely inhibit [Ca2+]i levels in activated mast cells. Similarly, CD40 siRNA (Supplemental Fig. 7) decreased [Ca2+]i levels in cocultured HMC-1 cells (Fig. 1B), whereas the IgG Ab control did not affect [Ca2+]i levels in HMC-1 cells or BMMCs (Supplemental Fig. 1C, 1D).

**Effects of anti-CD40 Ab on mediator releases in mast cells cocultured with astrocytes**

Ca2+ influx is essential for releasing proinflammatory mediators, such as histamine and LTs, during mast cell activation (23) and inducing some inflammatory cytokine production (30). Therefore, we examined the release of histamine and LTs in mast cells cocultured during the period of time indicated. Histamine release (in ng/106 cells) in cocultured HMC-1 cells was 51.3 ± 7.32 at 1 h, 93.4 ± 8.14 at 3 h, 161.2 ± 10.318 at 5 h, and 210.79 ± 16.32 at 12 h compared with basal levels of 23.0 ± 0.22. In cocultured BMMCs, histamine release (in ng/106 cells) was 109.5 ± 9.20 at 1 h, 209.5 ± 20.20 at 3 h, 271.3 ± 16.12 at 5 h, and 280.9 ± 11.19 at 12 h compared with controls (23.1 ± 0.25) (Fig. 1E).

LTs (in pg/106 cells) secreted in cocultured HMC-1 cells were 28.6 ± 4.10 at 5 h, 48.5 ± 2.50 at 12 h, and 47.4 ± 3.15 at 24 h compared with control (2.8 ± 0.30), and in cocultured BMMCs, amounts of LTs were 45.4 ± 1.30 at 5 h, 51.8 ± 1.65 at 12 h, and 56.8 ± 1.35 at 24 h compared with control (5.6 ± 0.90) (Fig. 1F).

Anti-CD40 Ab pretreatment decreased the histamine release (in ng/106 cells) by approximately 54% (25.2 ± 1.65 at 1 h; 42.7 ± 6.30 at 3 h; 87.8 ± 7.29 at 5 h; and 115.1 ± 8.52 at 12 h), compared with cocultured HMC-1 cells (74.3 ± 4.18 at 1 h; 178.7 ± 5.63 at 3 h; 192.1 ± 12.32 at 5 h; and 208.3 ± 13.12 at 12 h). In cocultured BMMCs, anti-CD40 Ab pretreatment also decreased the histamine release (in ng/106 cells) by approximately 54–70% (40 ± 9.60 at 1 h; 60 ± 14.60 at 3 h; 90.4 ± 17.26 at 5 h; and 134.7 ± 13.03 at 12 h), compared with cocultured BMMCs (99.5 ± 12.20 at 1 h; 199.5 ± 8.20 at 3 h; 261.3 ± 15.28 at 5 h; and 293.9 ± 18.93 at 12 h) (Fig. 1G). Anti-CD40 Ab showed similar inhibition of LT secretion (Fig. 1H).

**Effects of anti-CD40 Ab on the expressions of cytokines and chemokines in the mast cells cocultured with astrocytes**

We next measured cytokine and chemokine levels secreted in the media of HMC-1 and U87 cells cocultured for 12 h, as assessed by cytokine array kit (Supplemental Fig. 2). Coculture increased IL-6, TNF-α, MCP-1, MIG, and RANTES levels by 2.6-, 25.9-, 7.0-, 2.2-, and 3.4-fold, respectively. mRNA expression of IL-6, TNF-α, MCP-1, MIG, and RANTES peaked at 12 h in cocultured HMC-1 cells (Fig. 2A, left panel) and 6 h in BMMCs (Fig. 2A, right panel), as assessed by RT-PCR, as did protein levels (Supplemental Fig. 3). Anti-CD40 Ab pretreatment blocked this increase in cytokine mRNA levels in the cocultured HMC-1 cells (Fig. 2B, left panel) and BMMCs (Fig. 2B, right panel), as well as protein levels in HMC-1 cells (Supplemental Fig. 3).

**Effects of anti-CD40 Ab or CD40 siRNA on the activities of Rho family GTPases in the mast cells cocultured with astrocytes**

Rho family GTPases activate intracellular kinase cascades to modulate gene transcription (31). Coculture time dependently increased the activities of Rho family GTPases (Rac1/2, cdc42) in both mast cells (Supplemental Fig. 4A). Rac1, Rac2, and cdc42 activities peaked at 15, 15, and 30 min in cocultured HMC-1 cells (left panel) or BMMCs (right panel). Anti-CD40 Ab pretreatment blocked the increase in Rac1/2 and cdc42 activity in cocultured HMC-1 cells (Fig. 3A, left panel) and BMMCs (Fig. 3A, middle panel). Anti-CD40 Ab pretreatment also decreased the activities of the remaining GTPases (Supplemental Fig. 4B). Anti-CD40 Ab pretreatment also decreased the activities of the remaining GTPases (Supplemental Fig. 4B). Anti-CD40 Ab pretreatment also decreased the activities of the remaining GTPases (Supplemental Fig. 4B). Anti-CD40 Ab pretreatment also decreased the activities of the remaining GTPases (Supplemental Fig. 4B).
### Results

#### A. GST-Rac-1, GST-Rac-2, GST-cdc42, and Total Rac-1

- **Anti-CD40 Ab**
  - 15, 30 (min)
  - Control, Co-culture, CD40 siRNA, CD40 siRNA

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#### B. PKCα, p-PKCα, PKCβII, p-PKCβII

- **Anti-CD40 Ab**
  - 30, 60 (min)
  - Control, Co-culture, CD40 siRNA, CD40 siRNA

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#### C. ERK, p-ERK, JNK, p-JNK, p38, p-p38

- **Anti-CD40 Ab**
  - 60, 90 (min)
  - Control, Co-culture, CD40 siRNA, CD40 siRNA

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#### D. NF-κB, AP-1

- **Anti-CD40 Ab**
  - a, HMC 120, 30, 60, 90, 120 b (min)

<table>
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<th>Co-culture</th>
<th>CD40 siRNA</th>
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Effects of anti-CD40 Ab or CD40 siRNA on the activities of PKC isoforms in the mast cells cocultured with astrocytes

PKCa, βI, and βII activate JNK, MEKK2, and ERK5 (32). Therefore, we examined whether PKC isoforms (α, βI, βII, ζ, v, and θ/λ) are activated in both mast cells cocultured with both astrocytes. Coculture of HMC-1 cells or BMMCs increased phosphorylation of PKCa, βI, and βII, peaking at 30 min (Supplemental Fig. 4B), but did not change PKCζ and θ/λ phosphorylation (data not shown). Anti-CD40 Ab blocked this increase in PKCa, βI, and βII phosphorylation in HMC-1 cells (Fig. 3B, left panel) and BMMCs (Fig. 3B, middle panel). CD40 siRNA also inhibited PKC activation in cocultured HMC-1 cells (Fig. 3B, right panel).

Effects of anti-CD40 Ab or CD40 siRNA on the activities of MAPKs in the mast cells cocultured with astrocytes

The Ca²⁺/PKC pathways activate MAPKs to influence biological responses, including immune responses and proinflammatory cytokine expression (32). Coculture increased phosphorylation of ERK, JNK, and p38, peaking at 60 min in both cell types (Supplemental Fig. 4C). Anti-CD40 Ab inhibited activation of all three MAPKs in cocultured HMC-1 cells (Fig. 3C, left panel) and BMMCs (Fig. 3C, middle panel). CD40 siRNA inhibited the activities of MAPKs in cocultured HMC-1 cells (Fig. 3C, right panel).

To clarify cascades of PKC and MAPK, we used various inhibitors. PKC inhibitors (5 nM staurosporine and Gö6976) inhibited the phosphorylation of MAPK (Supplemental Fig. 5A, right panel) and PKC isoforms (Supplemental Fig. 5A, left panel). Although MAPK inhibitors (5 μM PD98059, 10 μM SP600125, 10 μM SB203580) inhibited MAPK activation (Supplemental Fig. 5B, right panel), they did not inhibit the phosphorylation of PKC isoforms (Supplemental Fig. 5B, left panel). These results suggest that MAPKs are downstream of PKC isoforms.

Effects of anti-CD40 Ab on the transcriptional factor activity in the mast cells cocultured with astrocytes

We next measured activation of the transcription factors, NF-κB and AP-1. Coculture increased NF-κB and AP-1 DNA binding in nuclear extracts. NF-κB DNA-binding activity peaked at 120 min, and AP-1 DNA-binding activity peaked at 90 min (Supplemental Fig. 4D). Anti-CD40 inhibited NF-κB and AP-1 activation in cocultured HMC-1 cells (Fig. 3D, left panel) and BMMCs (Fig. 3D, right panel).

Effects of a Rac inhibitor or Ca²⁺ influx inhibitor on Rho family and signaling molecules in the HMC-1 cells cocultured with astrocytes

The 8-oxo-dG is a Rac1/2 and cdc42 inhibitor (33–35). Rac1 increases Ca²⁺ influx in epithelial cells (36). The 8-oxo-dG pretreatment inhibited Rho activation (Fig. 4A) and [Ca²⁺]i levels (Fig. 4B), as well as PKC isoforms and MAPK downstream of

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FIGURE 3. Effects of anti-CD40 Ab or CD40 siRNA on the activation of mast cells cocultured with astrocytes. HMC-1 cells or BMMCs (3 × 10⁶ cells) were cocultured with U87 cells or primary brain astrocytes (1 × 10⁶ cells), respectively, for the indicated periods. Anti-CD40 Ab (300 ng/ml) was pretreated 1 h before coculture, and CD40 siRNA was performed, as described in Materials and Methods. We measured small G protein molecules, PKC isoforms, and MAPKs, and NF-κB activities were determined in protein extracts or nuclear extracts by GST pull-down assay, Western blot, and EMSA, respectively. A, Small GTPase activity. B, PKC isoform activity. C, Phosphorylation of MAPKs. D, NF-κB (upper panel) or AP-1 (lower panel) activities in cocultured HMC-1 cells (left panel) or in BMMCs (right panel). *Numbers below bands are the ratio of band density of each group versus those of control and total protein. The data are representative of four independent experiments (n = 4). a, negative control; anti-CD40 Ab, anti-CD40 Ab pretreatment; b, competition assay; BM, BMMC culture alone; coculture, cocultured HMC-1 cells; HMC, HMC-1 cell culture alone.
Ca\textsuperscript{2+} (Supplemental Fig. 6A, 6B). However, the Ca\textsuperscript{2+} influx inhibitor, 2-aminoethoxydiphenyl borate (2-APB) and inositol 1,4,5-triphosphate receptor antagonist, inhibited Ca\textsuperscript{2+} levels (Fig. 4B), but did not inhibit Rac1/2 and \textit{cdc42} activity (Fig. 4A). The 8-oxo-dG mimicked the effect of anti-CD40 Ab pretreatment and CD40 siRNA.

**Localization of mast cells in EAE mouse brain**

Mast cells are particularly abundant in the meninges, a common site of early inflammatory lesions in EAE (37). We therefore investigated the population of mast cells in EAE lesions (EAE score = 3.1 ± 0.10) using May-Grü newald-Giemsa (Fig. 5A). Mast cells in the EAE mouse brain increased 5-fold over controls.

Similarly, EAE increased the expression of the mast cell markers, \textit{c-kit} and CD40L, as well as astrocyte markers, GFAP and CD40, in the thalamus (Fig. 5B, 5C). Double labeling with \textit{c-kit} for mast cells (green) and GFAP for astrocytes (red) showed that mast cells colocalized with astrocytes in the thalamus (Fig. 5D).

Furthermore, we indicated schematic diagrams showing signaling pathways in the activation of mast cells cocultured with astrocytes (Fig. 6).
in vitro using both cell lines and primary cell cultures. Such interactions also play a role in CNS inflammatory disease, such as MS, and then we cocultured mast cells and astrocytes to study their effects.

Therefore, we hypothesized that interaction between mast cells and astrocytes plays an important role in CNS inflammatory disease, suggesting mast cells can mediate EAE (40), potentially exacerbating neurodegenerative diseases, such as MS (41), and that human mast cells stimulate the activated T cells that are in contact with them at the BBB (52). These reports support our results that mast cells activated with coculture induced preformed mediators (histamine) (Fig. 1E), mediator synthesis (LT) (Fig. 1F), and cytokines and chemokines (IL-6, TNF-α, MCP-1, MIG, and RANTES) (Fig. 2A, Supplemental Fig. 3), although interaction between mast cells and T cells was not observed in this experiment. Therefore, it can be inferred that the mediators secreted by mast cells activated by astrocytes may alter BBB permeability and induce demyelination in MS.

Astrocytes provide support for neuronal function in both healthy and inflamed CNS, including in MS (41). Astrocytes can exacerbate the myelin-specific immune response and inhibit remyelination (41). Mast cells share perivascular localization with astrocytes, and astrocytes support mast cell viability in vitro (19). Therefore, we hypothesized that interaction between mast cells and astrocytes plays an important role in CNS inflammatory disease, such as MS, and then we cocultured mast cells and astrocytes in vitro using both cell lines and primary cell cultures.

BBB breakdown is important in the development of new MS lesions (37). The integrity of the BBB can be affected by arachidonic acid and eicosanoids, bradykinin, histamine, serotonin, TNF-α, and free radicals (42–44), and by brain mast cell activation via acute restraint stress and corticotropic-releasing hormone (9, 10). However, histamine effect is controversial. Histamine can both improve clinical scores in EAE (46) and exacerbate it (47). Brain mast cell proteases elevated in the cerebrospinal fluid can contribute to myelin damage (demyelination), and mast cells are degranulated in response to myelin basic protein and substance P, leading to in vitro demyelination (48, 49). Therefore, it has been suggested that mast cells directly participate in the destruction of the myelin sheath in MS. Furthermore, the cytokines and chemokines expressed by mast cells may affect immune cell trafficking through direct chemotaxis and/or influence adhesion molecule expression on the endothelium. The ability of mast cells to migrate to secondary lymphoid organs also raises the possibility that they can regulate the induction and/or amplification of a polarized Th response (50). It has been reported also that murine mast cell- and TNF-dependent T cell activation can contribute to the progression and intensity of many different immune diseases, such as MS (51), and that human mast cells stimulate the activated T cells that are in contact with them at the BBB (52). These reports support our results that mast cells activated with coculture induced preformed mediators (histamine) (Fig. 1E), mediator synthesis (LT) (Fig. 1F), and cytokines and chemokines (IL-6, TNF-α, MCP-1, MIG, and RANTES) (Fig. 2A, Supplemental Fig. 3), although interaction between mast cells and T cells was not observed in this experiment. Therefore, it can be inferred that the mediators secreted by mast cells activated by astrocytes may alter BBB permeability and induce demyelination in MS.

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We found that cytokine-induced Ca\textsuperscript{2+} mobilization (Fig. 1B, 1C) and PKC (α, β, βII) activation (Fig. 3B), which are Ca\textsuperscript{2+}-dependent PKCs, and increased MAPK activity (Fig. 3C). Finally, coculture activated transcription factors (NF-kB, AP-1) (Fig. 3D) and produced cytokines and chemokines (Fig. 2A, Supplemental Fig. 3). Therefore, our data suggest that mast cells activated through coculture with astrocytes induce histamine and LT release via regulating Rho[Ca\textsuperscript{2+}]PKCs/MAPKs to induce production of cytokines and chemokines via NF-kB/AP-1 activities.

Anti-CD40 Ab pretreatment and CD40 siRNA blocked mediator release and signaling (small GTPases, [Ca\textsuperscript{2+}]\textit{i}, PKC isoforms, MAPKs). However, the anti-CD40 Ab or CD40 siRNA may not be able to completely block the interaction because anti-CD40 Ab may be degraded or other molecules may interact.

The IgG Ab as a control for the anti-CD40 Ab did not affect cocultures of mast cells (Supplemental Fig. 1C, 1D), eliminating a role for nonspecific Ab binding.

The absence of mast cells in the CNS does not completely prevent EAE (8, 13), in part because mast cells outside the CNS may influence the generation of the anti-MOG T cell response and direct T cell migration to target sites. That is, mast cells may disrupt the BBB by secreting chemotaxatrants that increase invasion of the T cells into CNS. However, in the rat EAE model, mast cells are increased 3-fold in the brain and significantly more are degranulated (60). We also observed that EAE increased the population of mast cells in the thalamus (Fig. 5A) as well as c-kit expression, a marker of mature mast cells. We also showed extensive expression of surface markers of mast cells and astrocytes (Fig. 5B, 5C) and their colocalization (Fig. 5D), although brain mast cells under physiological conditions have low FcRI and lack c-kit expression, unlike normal mast cells (61, 62). Our data suggest that mast cells and astrocytes in the thalamus may directly interact in close proximity during elevation of mast cell population. However, further work is needed to study these interactions.

In conclusion, mast cells are activated via interaction of CD40–CD40L in the surface of two adjacent cells, and the activated mast cells induce mediator release, such as histamine and LT, and cytokine production, via the Rho family and Ca\textsuperscript{2+}/PKC isoforms/MAPKs/NF-kB or AP-1 signal pathways (Fig. 6). That is, mast cells and astrocytes can occur in close proximity in brain. Mediators released in mast cells activated by astrocytes may contribute to the initiation of BBB destruction and demyelination in MS disease through constitutive/inducible CD40–CD40L interactions, providing a novel therapeutic target for neuroinflammatory diseases.

Disclosures
The authors have no financial conflicts of interest.

References
18. References.


