SCRG1 suppresses LPS-induced CCL22 production through ERK1/2 activation in mouse macrophage Raw264.7 cells

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Abstract. Recently, we identified the scrapie responsive gene 1 (SCRG1) secreted from mesenchymal stem cells (MSCs) and its receptor bone marrow stromal cell antigen 1 (BST1) as positive regulators of stem cell qualities such as self-renewal, migration abilities, and osteogenic differentiation potential. Here, we examined the effect of the paracrine activity of SCRG1 in macrophages. The mouse macrophage-like cell line Raw264.7 expressed BST1/β1 or BST1/β2 integrin as possible SCRG1 receptors. Unexpectedly, recombinant SCRG1 did not enhance cell proliferation, migration, or adhesion in these macrophages. However, further examination of the effect of SCRG1 in Raw264.7 cells did reveal a potent anti-inflammatory effect whereby SCRG1 suppressed LPS-induced CCL22 production. SCRG1 also induced the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in these cells and, moreover, a mitogen-activated protein kinase (MAPK)/ERK kinase inhibitor U0126 significantly suppressed the effect of SCRG1 on LPS-induced chemokine CCL22 production. Taken together, these data indicate that SCRG1 signals through the MAPK pathway and suppresses the LPS signaling pathway. CCL22 is generally known to be chemotactic for monocytes, dendritic cells, natural killer cells and chronically activated T lymphocytes, suggesting that MSC-derived SCRG1 may block infiltration of these cells. A mechanism is proposed by which MSCs play their immunosuppressive role through suppressing chemokine expression in monocyte/macrophage lineage cells.

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Introduction

Mesenchymal stem cells (MSCs) are adult stem cells with the ability to differentiate into mesenchymal tissue cells while retaining self-renewal and migration abilities (1). Many recent studies have revealed that MSCs possess immunomodulatory functions that they exert through cell-to-cell contacts, as well as by secreting growth factors, cytokines, and chemokines (2,3). The effect of immunosuppression with MSCs has been reported in graft-versus-host disease (4) and multiple system atrophy (5). MSCs have the ability to migrate to damaged tissue by inducing peripheral tolerance by inhibiting the release of pro-inflammatory cytokines (2). The advantages of MSC-based cell therapy have been demonstrated in acute lung injury (6), myocardial infarction (7), acute renal failure (8), cerebral ischemia (9) and Alzheimer's disease (10). At the cellular level, it has been shown that MSCs can directly inhibit both T lymphocyte and microglial cell proliferation and can negatively modulate the cytokine-secretion profile of dendritic cells and monocytes/macrophages (11-14).

In our recent study, we identified the scrapie responsive gene 1 (SCRG1) secreted from MSCs and its receptor complex bone marrow stromal cell antigen 1 (BST1)/β1 integrin, as positive regulators of stem cell qualities (15). SCRG1, which was identified by Dron *et al* as a protein that increased expression in the brain of scrapie infected mice, was shown to be associated with neurodegenerative changes in transmissible spongiform encephalopathy, as well as in brain injury, and is associated with autophagy (16-18). The SCRG1 gene encodes a 98-amino acid, cytokine-like peptide with an N-terminal signal peptide (19,20). Intriguingly, in stem cells SCRG1 was shown to maintain octamer-binding transcription factor 4 (Oct-4) and CD271/low-affinity nerve growth factor receptor (LNGFR) expression and thereby maintain the MSC's potential for self-renewal, migration abilities, as well as osteogenic differentiation potential, even at high stem cell passage numbers (15). Other cytokines and chemokines secreted from MSCs have been implicated in immunosuppression and repair of damaged tissues (21-24). MSC differentiation along different pathways is regulated by stimulation with various growth factors, cytokines, or chemokines, as has been demonstrated in the differentiation of bone marrow-derived MSCs (25,26).

SCRG1 secreted from MSCs is predicted to affect a variety of cell types *in vivo*. In this study, we hypothesized that SCRG1 secreted into the extracellular space by MSCs exhibits paracrine activity. To explore this possibility, we examined the paracrine effect of MSC-derived SCRG1 on the immune response of Raw264.7 macrophages. In particular, as a readout of macrophage function, we focused on macrophage production of CC-chemokine ligand 22 [CCL22; also known as MDC (macrophage-derived chemokine)], which is known to display chemotactic activity for monocytes, dendritic cells, natural killer cells, and chronically activated T lymphocytes (27-30).

Materials and methods

Reagents. Recombinant mouse SCRG1 (rmSCRG1), expressed in yeast, was purchased from MyBiosource, Inc. (MBS1177239, San Diego, CA, USA). The MAPK/ERK kinase inhibitor U0126 was purchased from Calbiochem (Merck Millipore, Darmstadt, Germany). Lipopolysaccharide (LPS) derived from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. Mouse macrophage-like Raw264.7 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in minimum essential medium Eagle's α-modification (αMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (HyClone, GE Healthcare Life Sciences, Logan, UT, USA) under the condition of 5% CO_2 at 37˚C.

Proliferation assay. Cell proliferation was analyzed by WST-1 assay reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Raw264.7 cells were cultured on 96-well plates (Nunc; Thermo Fisher Scientific, Waltham, MA, USA) in 100 µl complete medium containing with or without 100 ng/ml rmSCRG1. After five days, the cells were added with 10 µl WST-1 reagent and incubated for 1 h. The absorbance was measured using an MPR-A4i microplate reader (Tosoh Corp., Tokyo, Japan) at 450 nm.

Migration assay. The migration assay was performed using 8-µm pore sized Transwell cell culture inserts (BD Biosciences, Franklin Lakes, NJ, USA). Raw264.7 cells ($1.0x10^5$) were seeded on the upper well in 350 µl serum-free αMEM containing 0.1% BSA (Sigma-Aldrich). The lower well was filled in 600 µl complete medium containing with or without 100 ng/ml rmSCRG1. After incubation for 6 h, cells that had not migrated were scraped off with a cotton swab. The number of cells migrated to the lower side of the filter was stained with Diff-Quik Three-Step Stain Set (Sysmex, Kobe, Japan), and then counted using a microscope (Olympus IX70; Olympus Corp., Tokyo, Japan) under five high-power fields (x400 magnification).

Adhesion assay. Raw264.7 cells ($1.0x10^5$) were seeded onto a fibronectin-coated culture dish (BD Biosciences) and cultured in complete medium with or without 100 ng/ml rmSCRG1. After 6 h, non-adhered cells on the bottom of the dish were removed by washing twice with phosphate-buffered saline (PBS). The number of cells adhered to the culture dish were measured by the WST-1 assay described above. The absorbance of the dye in the culture directly correlates with the number of live cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Raw264.7 cells were either left unstimulated or stimulated with 10 ng/ml LPS, 100 ng/ml rmSCRG1, or 10 ng/ml LPS plus 100 ng/ml rmSCRG1 in the absence and presence of 1 mM U0126, for 6 h. Total RNA extraction, cDNA synthesis, RT-qPCR were performed with methods of our previous study (15). Expression of *Ccl22* was normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The following primer pairs were used; *Ccl22* (sense, 5'-GGCACCT ATCCAGTGCCACA-3' and antisense, 5'-TGGTGGACCAGC CTGAAACTC') and *Gapdh* (sense, 5'-TGTGTCCGTCGTGG ATCTGA-3' and antisense, 5'-TTGCTGTTGAAGTCGCAG GAG-3'). Relative expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method (31) as a fold-increase or -decrease.

Primer array. Raw264.7 cells were stimulated with 10 ng/ml LPS, 100 ng/ml rmSCRG1, or 10 ng/ml LPS plus 100 ng/ml rmSCRG1 for 6 h. Unstimulated cells were used as a control. Gene expression levels of a range of cytokines and chemokines were measured using a PrimerArray consisting of mouse cytokines and cytokine receptors (PN001, Takara Bio) and PrimerArray Analysis Tool version 2.0 (Takara Bio) according to the manufacturer's instructions. Genes whose expression levels increased more than 100-fold following LPS stimulation and less than 100-fold following SCRG1 treatment were identified.

Western blotting. Raw264.7 cells were serum-starved overnight and stimulated with 100 ng/ml rmSCRG1 for various period time. Western blotting was performed in our previously reported procedure (15). The following primary antibodies that has been purchased from Cell Signaling Technology (Danvers, MA, USA) were used; anti-p44/42 mitogen-activated protein kinase (ERK1/2), anti-phospho-ERK1/2, anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-Akt, anti-phospho-Akt, anti-focal adhesion kinase (FAK), anti-phospho-FAK. β-actin level measured were detected with an anti-β-actin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) as a loading control. The densitometry of the band measured by ImageJ version 1.44 software was expressed as the ratio of phosphorylation to the total molecule.

Enzyme-linked immunosorbent assay (ELISA). Raw264.7 cells were left unstimulated or were stimulated with 10 ng/ml LPS, 100 ng/ml rmSCRG1, or 10 ng/ml LPS plus 100 ng/ml rmSCRG1 for 48 h. The amount of secreted CCL22 in the culture medium was measured using a sandwich ELISA kit for mouse CCL22 (R&D Systems, Inc., Minneapolis, MN, USA). CCL22 levels were quantified according to the manufacturer's instructions.

Flow cytometry. A total of $1x10^5$ Raw264.7 cells suspended in PBS containing 2 mM EDTA and 0.5% FBS were incubated with either a phycoerythrin (PE)-conjugated anti-mouse BST1/CD157 (1:10), anti-mouse β1 integrin/CD29 (1:10), or anti-mouse β2 integrin/CD18 (1:10) (all from BioLegend, Inc.,

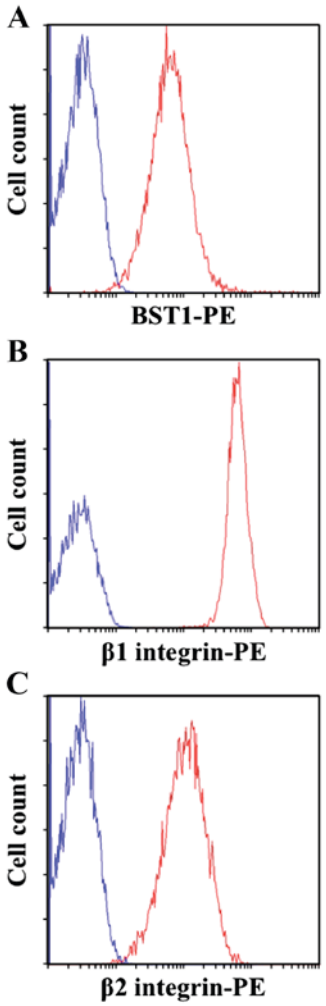

Figure 1. Raw264.7 cells express the bone marrow stromal cell antigen 1 (BST1), β1 integrin, and β2 integrin as a scrapie responsive gene 1 (SCRG1) receptor complex. Cell surface expression of BST1 (A), β1 integrin (B), and β2 integrin (C) was analyzed by flow cytometry with phycoerythrin (PE)-conjugated specific antibodies. Specific antibody staining (red) and isotype control IgG staining (blue) are shown.

San Diego, CA, USA) antibody for 1 h at 4˚C. Data acquisition and analysis was performed using a flow cytometer EPICS XL (Beckman Coulter, Brea, CA, USA).

Statistical analysis. All experiments were performed in triplicate. Numerical data were presented as the mean ± standard deviation (SD), and significant differences were analyzed by Student's *t*-test. $P<0.05$ were considered statistically significant.

Results

Raw264.7 cells express BST1, and β1 and β2 integrins as an SCRG1 receptor complex. Our recent study revealed that SCRG1 secreted from MSCs forms a complex with the membrane proteins BST1 and β1 integrin, which acts as a receptor for its autocrine/paracrine activity (15). Further evidence for this novel receptor was provided by Lavagno *et al*, which showed BST1 also interacts with β1 and β2 integrins at the neutrophil cell surface (32). Based on these data, we investigated the expression of BST1, β1 integrin, and β2 integrin in mouse macrophage-like Raw264.7 cells by flow cytometry. As shown in Fig. 1, Raw264.7 cells co-expressed BST1, β1 integrin, and β2 integrin on the cell surface. These results indicate that Raw264.7 cells express the SCRG1 receptor complex.

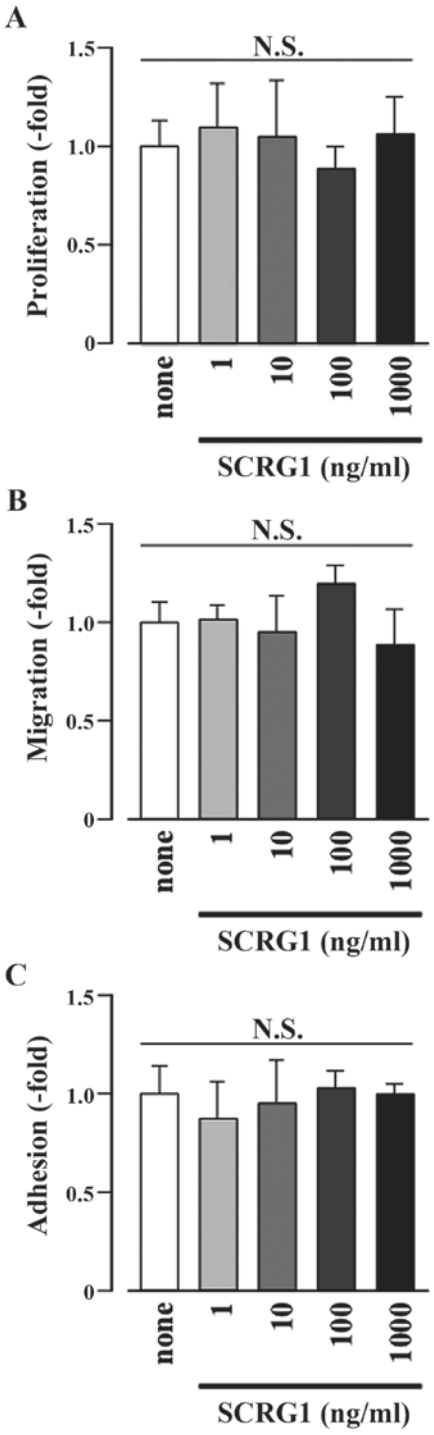

Figure 2. Scrapie responsive gene 1 (SCRG1) did not affect cell proliferation, migration, or adhesion of Raw264.7 cells. Cell proliferation (A), migration (B), and adhesion (C) for Raw264.7 cells were left unstimulated (none) or were stimulated with SCRG1 (1-1,000 ng/ml). The results are expressed as the fold change relative to the respective control (none). Data are presented as the means ± SD. *$P<0.05$ vs. unstimulated control within each cell. N.S., not significant.

SCRG1 does not affect the statuses of cell proliferation, migration, and adhesion of Raw264.7 cells. In MSCs, SCRG1

Table I. Genes whose expression increased more than 100-fold after LPS treatment and less than 100-fold by SCRG1 treatment alone relative to unstimulated Raw264.7 cells.

Gene symbol	Gene name	Fold change relative to unstimulated Raw 264.7 cells		
		LPS	LPS+SCRG1	SCRG1
Ccl22	Chemokine (C-C motif) ligand 22	4420.5	1520.1	96.335
Ccl8	Chemokine (C-C motif) ligand 8	2856.4	1770.5	0.84089
Cxcl11	Chemokine (C-X-C motif) ligand 11	1746.1	843.35	89.884
Ccl12	Chemokine (C-C motif) ligand 12	929.29	749.61	70.521
Csf1	Colony stimulating factor 1	206.50	125.36	17.876
Cx3cl1	Chemokine (C-X3-C motif) ligand 1	154.34	115.36	1.3755

LPS, lipopolysaccharide.

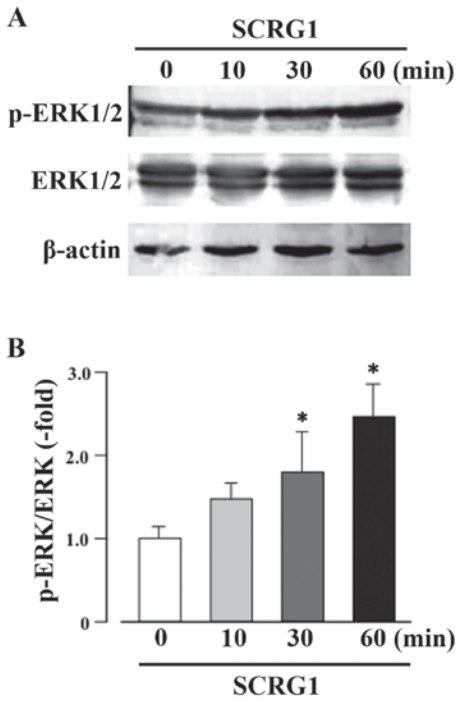

Figure 3. Scrapie responsive gene 1 (SCRG1) enhances the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in Raw264.7 cells. (A) ERK1/2 phosphorylation was measured using western blotting with anti-phospho-ERK1/2 (p-ERK1/2) antibodies in Raw264.7 cells stimulated with 100 ng/ml SCRG1. Total ERK1/2 levels were measured using a primary anti-ERK1/2 (ERK1/2) antibody. As a loading control β-actin levels were measured using an anti-β-actin antibody. (B) Densitometry analysis of band intensity in western blotting was expressed as the ratio of phosphorylation to the total molecule. *P<0.05, statistically significant vs. unstimulated control.

promotes cell migration through the β1 integrin/focal adhesion kinase (FAK) -dependent phosphoinositide 3-kinase (PI3K)/Akt pathway (15). Here, to understand the biological processes controlled by SCRG1 in macrophages, the effects of SCRG1 on cell proliferation, migration, as well as adhesion activity were investigated. As shown in Fig. 2, rmSCRG1 did not affect proliferative, migratory, or adhesive activities in Raw264.7 cells. Thus, these results indicate that the bioactivity of SCRG1 in monocyte/macrophage lineage cells is different from that in MSCs.

SCRG1 enhances the phosphorylation of ERK1/2 in Raw264.7 cells. The MAPK pathway, in conjunction with the nuclear factor-κB (NF-κB) pathway, is closely associated with the macrophage immune response (33,34). Accordingly, the intracellular signaling pathways induced by SCRG1 in Raw264.7 cells were investigated. Treatment of cell for 30 min with rmSCRG1 significantly enhanced the phosphorylation of ERK1/2 (Fig. 3). In contrast, phosphorylation of FAK, Akt, SAPK/Jun amino-terminal kinase (JNK), or p38 MAPK by rmSCRG1 treatment in Raw264.7 cells was not observed (data not shown). These results indicate that SCRG1 specifically induces the activation of the ERK1/2 pathway.

SCRG1 suppresses LPS-induced CCL22 production through the activation of ERK1/2 in Raw264.7 cells. By primer array analysis, we next investigated the effects of SCRG1 on the expression of LPS-induced chemokines and cytokines in Raw264.7 cells. As shown in Table I, rmSCRG1 suppresses the LPS-induced production of five chemokines and one cytokine in these cells. In particular, LPS-induced *Ccl22* expression was reduced to less than half by rmSCRG1 treatment. Following on from this, we examined the association between suppression of CCL22 expression and ERK activation by rmSCRG1 in Raw264.7 cells. Both the LPS-induced increases in CCL22 mRNA expression, and protein secretion were significantly suppressed by rmSCRG1 treatment in Raw264.7 cells (Fig. 4). In addition, this suppressive effect of rmSCRG1 was completely abolished by treatment with the MAPK/ERK kinase inhibitor, U0126. These results indicate that LPS-induced CCL22 production in macrophages was suppressed by treatment with SCRG1 through the activation of an ERK1/2-mediated signal.

Discussion

Several roles of SCRG1 suggested in this study and our previous studies are shown in Fig. 5. Recently, we identified a novel ligand-receptor combination, SCRG1/BST1, that maintains expressions of the stem cell markers Oct-4 and CD271/LNGFR in MSC, as well as self-renewal, migration, and osteogenic differentiation potential during *ex vivo* expansion (15). Here, we investigated the expression of the SCRG1

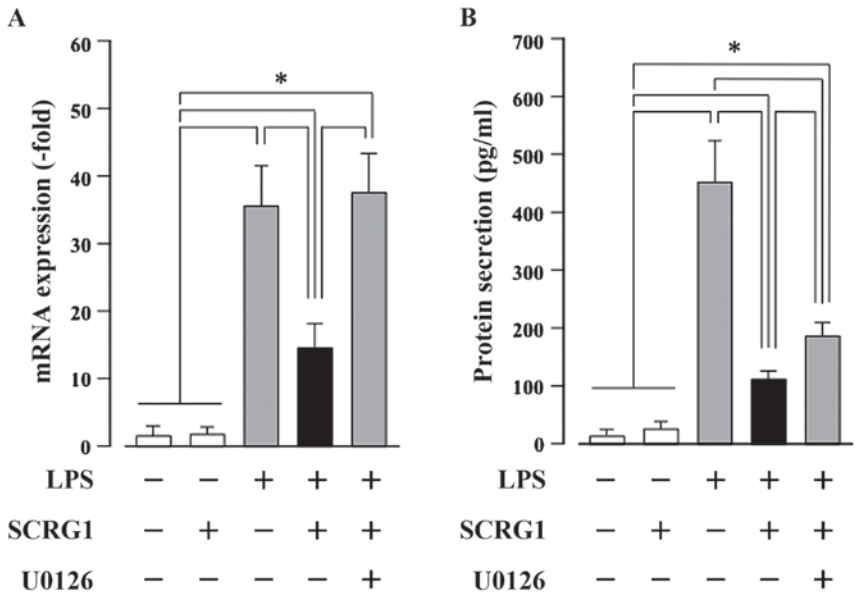

Figure 4. Scrapie responsive gene 1 (SCRG1) suppresses lipopolysaccharide (LPS)-induced CC-chemokine ligand 22 (CCL22) production through the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in Raw264.7 cells. Raw264.7 cells were stimulated with or without 10 ng/ml LPS, 100 ng/ml rmSCRG1, and 1 mM U0126 for either 6 h (A) or 48 h (B). (A) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed with specific oligonucleotide primers. mRNA expression level of *Ccl22* was normalized to glyceraldehyde-3-phosphate dehydrogenase, and the results are expressed as the fold change relative to the unstimulated control. (B) The amount of secreted CCL22 in the culture medium was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) for mouse CCL22. In (A) and (B), data are presented as the means ± SD. *P<0.05 vs. unstimulated control within each cell.

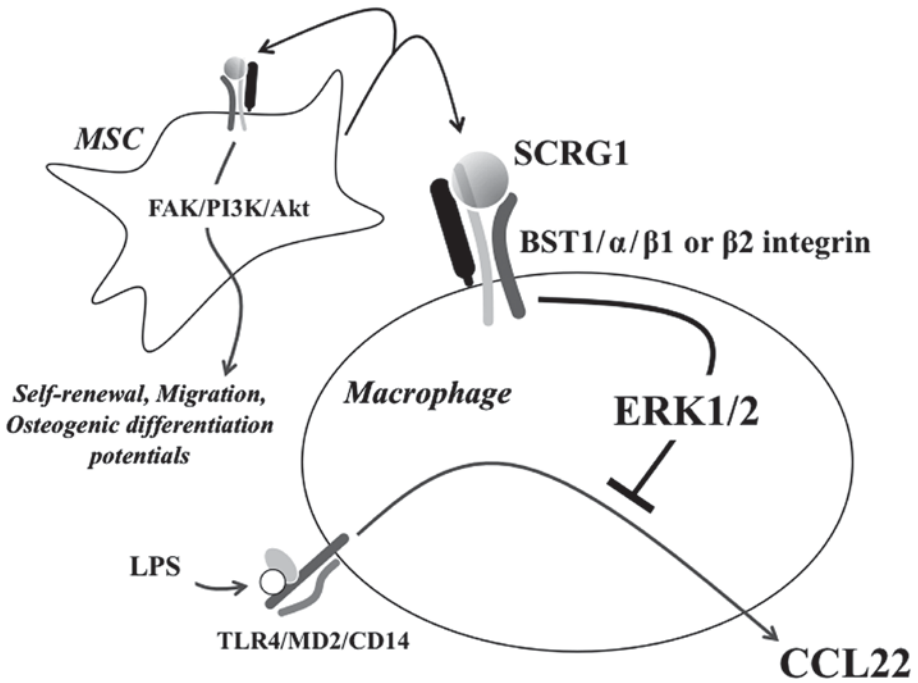

Figure 5. Several roles of scrapie responsive gene 1 (SCRG1) suggested in this study and our previous reports. SCRG1 is hypothesized to be secreted into the extracellular space by mesenchymal sem cells (MSCs) and to have autocrine/paracrine activity. The putative receptor for SCRG1 is a complex of bone marrow stromal cell antigen 1 (BST1) and β1 or β2 integrins on the cell surface. The SCRG1/BST1 axis positively regulates the self-renewal, migration, and osteogenic differentiation potentials of MSCs through the FAK/PI3 K/Akt signaling pathway. In macrophage, lipopolysaccharide (LPS) interacts with a heterologous receptor involving toll-like receptor 4 (TLR4), CD14, and MD2. SCRG1 suppresses LPS-induced CC-chemokine ligand 22 (CCL22) production of macrophage in a extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent manner.

receptor components, BST1, β1 integrin, and β2 integrin in mouse macrophage-like Raw264.7 cells. In addition to BST1, these cells also expressed β1 and β2 integrins (Fig. 1). BST1 is an ectoenzyme with a glycosyl phosphatidylinositol anchor and a NADase/ADP-ribosyl cyclase activity that belongs to the CD38 family (35,36). It has been found on the cell surface of stromal (37) and bone marrow-derived cells (38), and it facilitates pre-B-cell growth and induces cell migration (39). Under

the condition of a complex of BST1 with either β1 integrin or β2 integrin, BST1 has been shown to promote the phosphorylation of FAK through the use of an agonistic monoclonal antibody (32,40,41). Furthermore, it has also been reported that BST-1 regulates the adhesion and migration of leukocytes via phosphorylation of Akt and MAPKs (42,43). In this study, we also observed that SCRG1 enhanced the phosphorylation of ERK1/2 in Raw264.7 cells (Fig. 3).

Notably, SCRG1 did not enhance cell proliferation, migration, or adhesion of Raw264.7 cells (Fig. 2), indicating a different biological function in these cells. In support of this, we demonstrated that SCRG1 suppresses LPS-induced CCL22 production in these mouse macrophage-like Raw264.7 cells in a MAPK/ERK-dependent manner (Table I, Fig. 4). LPS and other microbial products are recognized by the host's toll-like receptors (TLRs) family members (44). LPS interacts with a heterologous receptor involving TLR4 (45,46), CD14 (47,48) and MD2 (49-51). By LPS binds to the TLR4, two major signaling pathways via the adapter molecules TIR-domain-containing adaptor inducing IFN-β (TRIF) or myeloid differentiation factor 88 (MyD88) are activated (45,52-55). Significantly, both the Myd88 and TRIF pathways result in activation of the transcription factor, NF-κB, a central regulator of the LPS response, which induces cytokine and chemokine production as well as stress responses in many cell types, including macrophages (53,54). NF-κB is involved in regulating the expression of multiple genes involved in inflammatory and immune responses (56). Innate and adaptive immune response are mainly regulated by MAPKs, including ERK, JNK, and p38 MAPK, in addition to the NF-κB pathway. In macrophages, activation of the MEK/ERK pathway by bacterial infection regulates various inflammatory responses (57-61). The MEK/ERK pathway is one of the most studied intracellular signaling pathways in monocyte-derived macrophages activated by LPS-induced pro-inflammatory responses (62-64). In addition, findings have been previously reported that NF-κB-dependent gene expression is controlled by the activation of ERK1/2 without affecting DNA binding (65-69). Therefore, our results indicate that the SCRG1-induced ERK1/2 signaling pathway controls NF-κB-dependent chemokine CCL22 expression.

CCL22, a member of the CC chemokine families, is mainly produced by monocyte-derived macrophages, mast cells, and inflammatory dendritic cells upon stimulation with microbial products (70,71). CCL22 binds to its receptor CCR4 plays an important homeostatic role in leukocyte trafficking, activation of innate immune cells, Th2 immunopathology, as well as accumulation of regulatory T (Treg) cells in solid tumor (72-74). CCL 22 also plays a role in recruiting Treg cells into synovial fluid in inflammatory diseases like rheumatoid arthritis (75). CCL22 plays an important role in a variety of other diseases, including allergic rhinitis (76), atopic dermatitis (77), and lymphoma (78). Recent studies suggested that CCL22 can be used as a biomarker for autoimmune diseases (79). On the other hand, the role of CCL 22 in regulation of immune homeostasis is unclear (80,81).

In conclusion, here we clearly demonstrate that SCRG1, which *in vivo* could be derived from MSCs, suppresses LPS-induced chemokine CCL22 production in Raw264.7 macrophages. The mechanism appears to involve the MAPK ERK1/2 pathway, since SCRG1 induced the phosphorylation of ERK1/2 and a MAPK/ERK kinase inhibitor U0126 ablated the suppressive effect of SCRG1 on LPS-induced chemokine CCL22 production. These results suggest a model whereby MSCs play their immunosuppressive role by secreting SCRG1, which then suppresses microbial products-induced chemokine expression in monocyte/macrophage lineage cells in a paracrine fashion. We have additionally established fluorescently tagged immortalized MSC lines derived from different tissues of GFP- and tdTomato-transgenic mice (25,82). These cell lines can be used for *in vivo* imaging analyses on proliferation and differentiation of MSCs, as well as *in vivo* imaging studies to test cell therapies and regenerative medicine techniques, providing insight into diseases such as bone and immune disorders, fibrosis, and cancer progression or metastasis. Our findings provide new insights into the molecular mechanisms of MSCs, as well as a novel perspective for understanding the immune regulatory mechanisms of MSCs.

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