LECT2 protects mice against bacterial sepsis by activating macrophages via the CD209a receptor

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Leukocyte cell–derived chemotaxin 2 (LECT2) is a multifunctional cytokine and reduced plasma levels were found in patients with sepsis. However, precise functions and mechanisms of LECT2 remain unclear. The aim of the present study was to determine the role of LECT2 in modulating immune responses using mouse sepsis models. We found that LECT2 treatment improved outcome in mice with bacterial sepsis. Macrophages (MΦ), but not polymorphonuclear neutrophils, mediated the beneficial effect of LECT2 on bacterial sepsis. LECT2 treatment could alter gene expression and enhance phagocytosis and bacterial killing of MΦ in vitro. CD209a was identified to specifically interact with LECT2 and mediate LECT2-induced MΦ activation. CD209a-expressing MΦ was further confirmed to mediate the effect of LECT2 on sepsis in vivo. Our data demonstrate that LECT2 improves protective immunity in bacterial sepsis, possibly as a result of enhanced MΦ functions via the CD209a receptor. The modulation of MΦ functions by LECT2 may serve as a novel potential treatment for sepsis.

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Abbreviations used: CLP, cecal ligation and puncture; Co-IP, coimmunoprecipitation; CRD, carbohydrate recognition domain; hpi, hours post injection; LECT2, leukocyte cell–derived chemotaxin 2; MΦ, macrophages; PMN, polymorphonuclear neutrophil; RT-qPCR, real-time quantitative PCR.

Sepsis is defined as infection with systemic inflammatory reaction syndrome and can be the result of injury, burn, pancreatitis, surgery, and other disease states (Levy et al., 2003). More than half of sepsis cases are caused by infection with bacteria, among which the most important pathogens include *Escherichia coli* and *Pseudomonas aeruginosa* (Annane et al., 2005). Sepsis remains the leading cause of death in critically ill patients worldwide, despite modern advances in critical care. Macrophages (MΦ) are the key component of the innate immune system, forming a bridge between innate and adaptive immunity by producing a myriad of cytokines, and phagocytosing and presenting antigens to the immune system, responses which are severely impaired in septic patients (Hotchkiss and Karl, 2003). Thus, MΦ are a potentially important therapeutic target in sepsis (Anderson et al., 2012).

Leukocyte cell–derived chemotaxin 2 (LECT2) is a multifunctional factor originally identified as a neutrophil chemotactic protein (Yamagoe et al., 1996), consisting of 151 amino acids and three intramolecular disulfide bonds. It is produced in the liver and secreted into the blood. LECT2 is involved in many pathological conditions, such as renal amyloidosis (Benson et al., 2008), hepatocarcinogenesis (Ong et al., 2011), and severe liver injury (Saito et al., 2004). Most recently, plasma LECT2 levels were found to be down-regulated in septic patients (Ando et al., 2012), suggesting a relationship between LECT2 and sepsis. However, precise functions and mechanisms of LECT2

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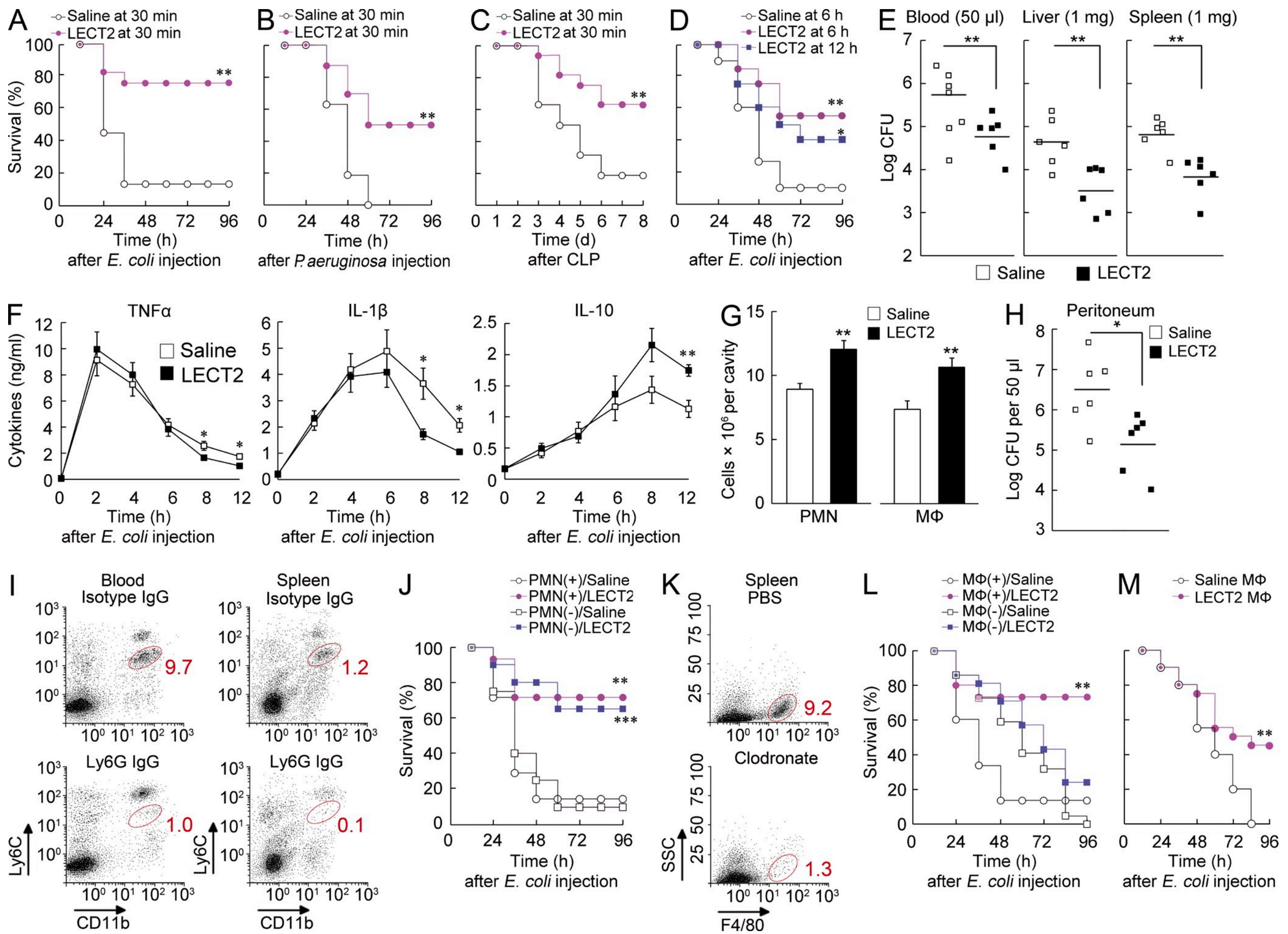

Figure 1. LECT2 improves outcome in experimental sepsis by activating MΦ. (A–C) Mice were treated with LECT2 during *E. coli*–induced sepsis ($n = 16$ per group; A), *P. aeruginosa* sepsis ($n = 16$ per group; B), and CLP sepsis ($n = 16$ per group; C). (D) Survival at 6 or 12 h after *E. coli* challenge ($n = 20$ per group). (E and F) Bacterial clearance ($n = 5$ per group; E) and plasma cytokine expression ($n = 4$ per group; F) in mice after induction of *E. coli* sepsis. (G) Numbers of PMN and MΦ were analyzed in the peritoneum of *E. coli*–injected mice treated with saline or LECT2 ($n = 5$ per group). (H) Bacterial clearance in the peritoneum of *E. coli*–injected mice ($n = 6$ per group). (I) Flow cytometry plots show $Ly6C^{int}$ $CD11b^{+}$ PMN in the circulation isolated from blood and spleens at day 2 after Ly6G antibody treatment ($n = 6$ per group). (J) Depletion of PMN followed by LECT2 on survival of mice with *E. coli*–induced sepsis (PMN(+)/Saline $n = 14$; PMN(+)/LECT2 $n = 14$; PMN(−)/Saline $n = 20$; PMN(−)/LECT2 $n = 20$). PMN(+) denotes normal mice; PMN(−) denotes PMN-depleted mice. (K) Flow cytometry plots show F4/80^{+} MΦ in the circulation isolated from spleens at day 2 after clodronate liposomes treatment ($n = 6$ per group). (L) Mortality after MΦ depletion and treatment with LECT2 or saline in mice with *E. coli*–induced sepsis (MΦ(+)/Saline $n = 15$; MΦ(+)/LECT2 $n = 15$; MΦ(−)/Saline $n = 22$; MΦ(−)/LECT2 $n = 21$). MΦ(+) denotes normal mice; MΦ(−) denotes MΦ-depleted mice. (M) Survival after transfer of LECT2-treated MΦ in mice with *E. coli*–induced sepsis ($n = 20$ per group). Horizontal bars indicate means (E and H). Error bars indicate SEM. Data are representative of two (A–F and I–M) and three (G and H) independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

in sepsis remain unclear. C-type lectin receptors (CLRs) perform multiple functions in myeloid cells, including MΦ (Kang et al., 2003; Osorio and Reis e Sousa, 2011). CD209, pattern recognition receptors belonging to the CLR superfamily, can not only recognize exogenous carbohydrate ligands to provide innate resistance to microbial infection (Robinson et al., 2006) but also bind to endogenous self-ligands to maintain immune homeostasis (García-Vallejo and van Kooyk, 2009). For example, SIGN-R1(CD209b) in spleen MΦ can capture pneumococcal capsular polysaccharide to activate complement system through an unusual C3 activation pathway (Kang et al., 2006).

In this study, we determined that LECT2 treatment improved survival in septic mice via the increased phagocytic ability, bactericidal activity, and beneficial cytokine production of MΦ. These effects were mediated through CD209a. Moreover, CD209a-expressing MΦ mediated the effect of LECT2 on sepsis. Collectively, our data suggest that LECT2 plays a potentially important role in MΦ activation and for the treatment of sepsis.

RESULTS AND DISCUSSION

LECT2 protects mice against bacterial sepsis through MΦ

We used a mouse model of *E. coli* sepsis to examine the relationship between plasma LECT2 levels and septic survival.

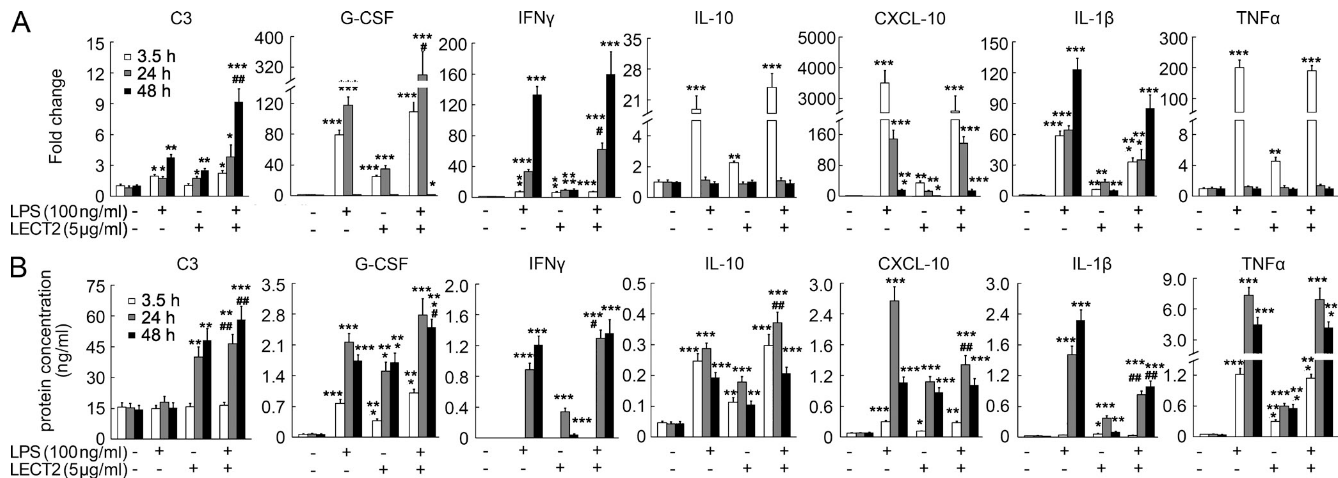

Figure 2. LECT2 alters the gene expression in mouse MΦ. (A) In resting or LPS-stimulated MΦ, RT-qPCR was used to measure the mRNA levels of C3, G-CSF, IFN-γ, IL-10, CXCL-10, IL-1β, and TNF after treatment with LECT2. (B) In resting or LPS-stimulated MΦ, ELISA assays were used to measure protein secretion after treatment with LECT2. Error bars indicate SEM. Data are representative of at least three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus negative control group at same time point. #, P < 0.05; ##, P < 0.01 versus LPS group.

At 12 h post injection (hpi), plasma LECT2 levels in septic mice (18.62 ± 1.10 ng/ml, *n* = 20) were significantly lower compared with control (46.88 ± 4.94 ng/ml, *n* = 6; P < 0.001). Plasma LECT2 levels in mice that died by 24 hpi (15.21 ± 1.03 ng/ml, *n* = 10) were significantly lower than those in mice that survived (22.02 ± 1.22 ng/ml, *n* = 10; P < 0.001). Plasma levels of LECT2 in mice that recovered from sepsis by 96 hpi (44.05 ± 2.83 ng/ml, *n* = 4) was similar to control.

We subsequently generated recombinant mouse LECT2 to investigate whether treatment with LECT2 would improve survival in bacterial sepsis models. The survival of mice was increased after treatment with LECT2 when compared with saline-treated control animals in a model of *E. coli* sepsis, *P. aeruginosa* sepsis, and after cecal ligation and puncture (CLP; Fig. 1, A–C). For example, the survival of LECT2-treated mice was 75.0% and that of saline-treated mice was only 12.5% in *E. coli* sepsis. We next asked whether delayed administration of LECT2 could still protect in conditions of already established sepsis. Kinetic studies showed that LECT2 still exerted a significant effect when administered 12 h after *E. coli* injection (Fig. 1 D). Sepsis is characterized by inefficient bacterial clearance and the overexpression of inflammatory cytokines (Cohen, 2002). Therefore, we examined the effects of LECT2 treatment on the bacterial burden and cytokine production during *E. coli* sepsis. The blood, liver, and spleen samples from saline-treated mice contained a higher bacterial burden than the samples from LECT2-treated mice (Fig. 1 E). LECT2 treatment did not alter the mouse plasma levels of TNF, IL-1β, and IL-10 at 0, 2, 4, and 6 h, decreased the plasma levels of TNF and IL-1β at 8 and 12 h, and increased the plasma levels of IL-10 at 12 h (Fig. 1 F). We further investigated the cellular mechanism of LECT2 on immune cells. LECT2 acts as a chemoattractant to both polymorphonuclear neutrophils (PMNs) and MΦ but not to T or B cells (unpublished data). The PMN and MΦ infiltration in the peritoneum after *E. coli* injection was next investigated. The numbers of infiltrating PMN and MΦ were significantly augmented in LECT2-treated mice, resulting in a 1.34- and 1.42-fold increase, relative to the saline-treated mice, respectively (Fig. 1 G). In the peritoneum, the bacterial burden in saline-treated mice was 33-fold higher than that in LECT2-treated mice (Fig. 1 H). Thus, we hypothesized that PMN and/or MΦ might mediate the beneficial effect of LECT2 on sepsis, and related experiments were done next. We first assessed the importance of PMN and MΦ in the survival of LECT2-treated septic mice using cell depletion. There was no significant change in the survival of LECT2-treated septic mice after the depletion of PMN (Fig. 1, I and J). In contrast, depleting MΦ dramatically impaired the survival of septic mice treated with LECT2 (Fig. 1, K and L), suggesting that MΦ may associate with the beneficial effect of LECT2 in sepsis. Second, we assessed the role of MΦ in LECT2-treated sepsis using adoptive cell transfer. The survival of septic mice receiving LECT2-treated MΦ was 45%, whereas septic mice receiving saline-treated MΦ all died (Fig. 1 M). These results suggest that the improved outcomes after LECT2 treatment of sepsis are mediated mainly by MΦ.

Recent clinical data have shown that higher plasma levels of LECT2 might predict a favorable outcome in human bacterial sepsis (Ando et al., 2012). Our results showed that LECT2 treatment effectively improved the outcome in mouse models of sepsis. LECT2 treatment did not suppress early inflammation but improved protective immune response in septic mice. The PMN and MΦ both possess immunomodulatory ability in sepsis (Alves-Filho et al., 2010; Anderson et al., 2012). In MΦ-depleted mice, the effect of LECT2 on survival of septic mice was no longer seen, whereas the beneficial effect of LECT2 on sepsis was still significant in PMN-depleted mice. Therefore, it appears that MΦ is a valid target for LECT2 in treatment of sepsis.

LECT2 can directly affect mouse MΦ

We first determined preferentially expressed genes in LECT2-treated peritoneal MΦ using microarray analysis and identified cytokine–cytokine receptor interactions and phagosome as the top-ranking pathways after 3.5 and 48 h of treatment, respectively. It suggests that LECT2 may alter cytokine production and phagocytosis in MΦ.

According to microarray data, real-time quantitative PCR (RT-qPCR) was performed to confirm the effect of LECT2 on mRNA expression of several up-regulated genes in LPS-stimulated MΦ at different time points (Fig. 2 A). In resting MΦ, all tested genes were found to be up-regulated after treatment with LECT2 (Fig. 2 A). In LPS-stimulated MΦ, treatment with LECT2 resulted in increased expression of G-CSF (up to 2.6-fold) and IFN-γ (up to 1.9-fold) at 24 h and C3 (up to 2.5-fold) at 48 h as compared with controls (Fig. 2 A). We also confirmed increased protein secretion in resting or LPS-stimulated MΦ treated with LECT2 at the different time points using ELISAs (Fig. 2 B). The effect of LECT2 on phagocytic ability of MΦ was investigated by monitoring uptake of *E. coli*–FITC and FluoSpheres. Treatment with LECT2 for 48 h resulted in a significant increase in the MΦ uptake of *E. coli*–FITC (up to 2.0-fold) and FluoSpheres (up to 2.3-fold; Fig. 3 A) and was also supported by the result of a flow cytometry assay (Fig. 3 B). Moreover, the direct measurement of CFUs showed that survival of intracellular bacteria in LECT2-treated MΦ was only 32.5% of control MΦ. Because C3 is dramatically induced by LECT2 and pivotal for labeling of the target particles to be taken up by phagocytes (Gros et al., 2008), we determined the correlation between C3 and LECT2-enchanced phagocytosis of MΦ. Treating resting MΦs with the supernatants from LECT2-treated MΦ (with 40.1 ng/ml C3) significantly enhanced the phagocytosis in comparison to the controls (with 15.3 ng/ml C3; Fig. 3 C). C3 and the type 3 complement receptor (CR3) blockade, by adding neutralizing antibodies, significantly decreased the phagocytosis in LECT2-treated MΦ (Fig. 3, D and E). These results suggest that LECT2-mediated phagocytosis enhancement is at least in part via C3/CR3 pathway.

Sepsis will result in the impairment of phagocytosis, bacterial killing, and cytokine production in tissue MΦ (Hotchkiss and Karl, 2003). Our results indicated that LECT2 directly activated MΦ, resulting in increased phagocytic and bacterial killing activities. LECT2 also induced the up-regulated expression of a panel of cytokines, among which G-CSF, IFN-γ, and CXCL-10 are known to play beneficial roles in sepsis (Hotchkiss and Karl, 2003; Kelly-Scumpia et al., 2010; Garg et al., 2012). Based on these results, we conclude that LECT2 improves outcomes during sepsis by activating MΦ effector functions.

LECT2 activates mouse MΦ through CD209a

Yeast two hybrid (Y2H) screening of an MΦ cDNA library revealed that LECT2 interacted with carbohydrate recognition domain (CRD; amino acids 103–238) of CD209a. CD209a is highly structurally related to CD209b, the most extensively studied mouse homologue of human DC-SIGN (Taylor et al., 2004; Powlesland et al., 2006; Gonzalez et al., 2010). The interaction between mouse LECT2 and the CRD of CD209a was confirmed by coimmunoprecipitation (Co-IP)

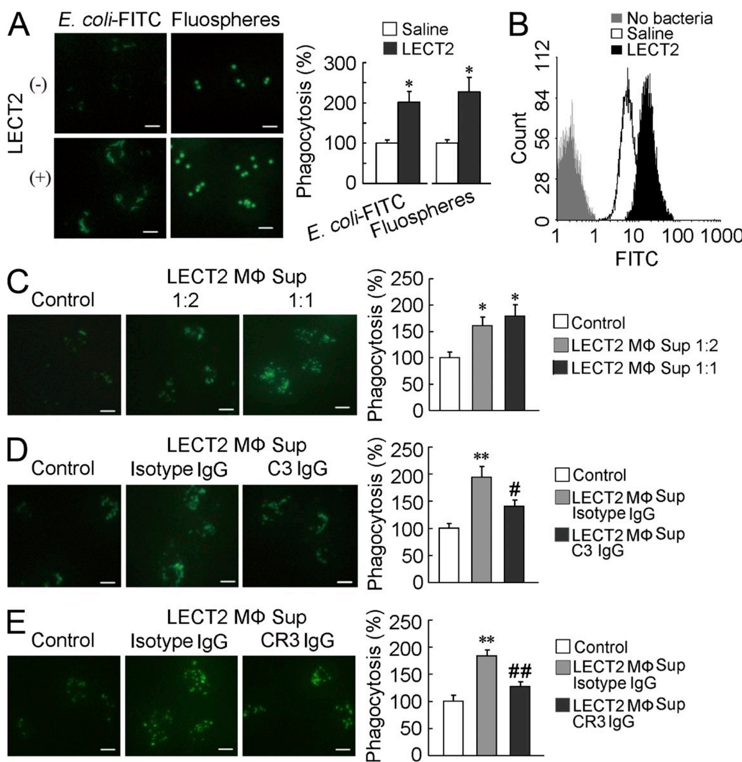

Figure 3. Effect of LECT2 on phagocytosis of mouse MΦ. (A) Uptake of *E. coli*–FITC or FluoSpheres with LECT2 treatment. (B) Flow cytometry histogram represents the *E. coli*–FITC phagocytosis by MΦ. (C) Uptake of *E. coli*–FITC by resting MΦ treated with LECT2 supernatant. *, P < 0.05. (D) C3-mediated LECT2 induction of MΦ phagocytic function. (E) Participation of CR3 in LECT2 induction of MΦ phagocytic function. Error bars indicate SEM. Data are representative of at least six (A), three (B), and four (C–E) independent experiments. Bars, 10 µm. Sup, supernatants. **, P < 0.01 versus control. #, P < 0.05; ##, P < 0.01 versus isotype IgG.

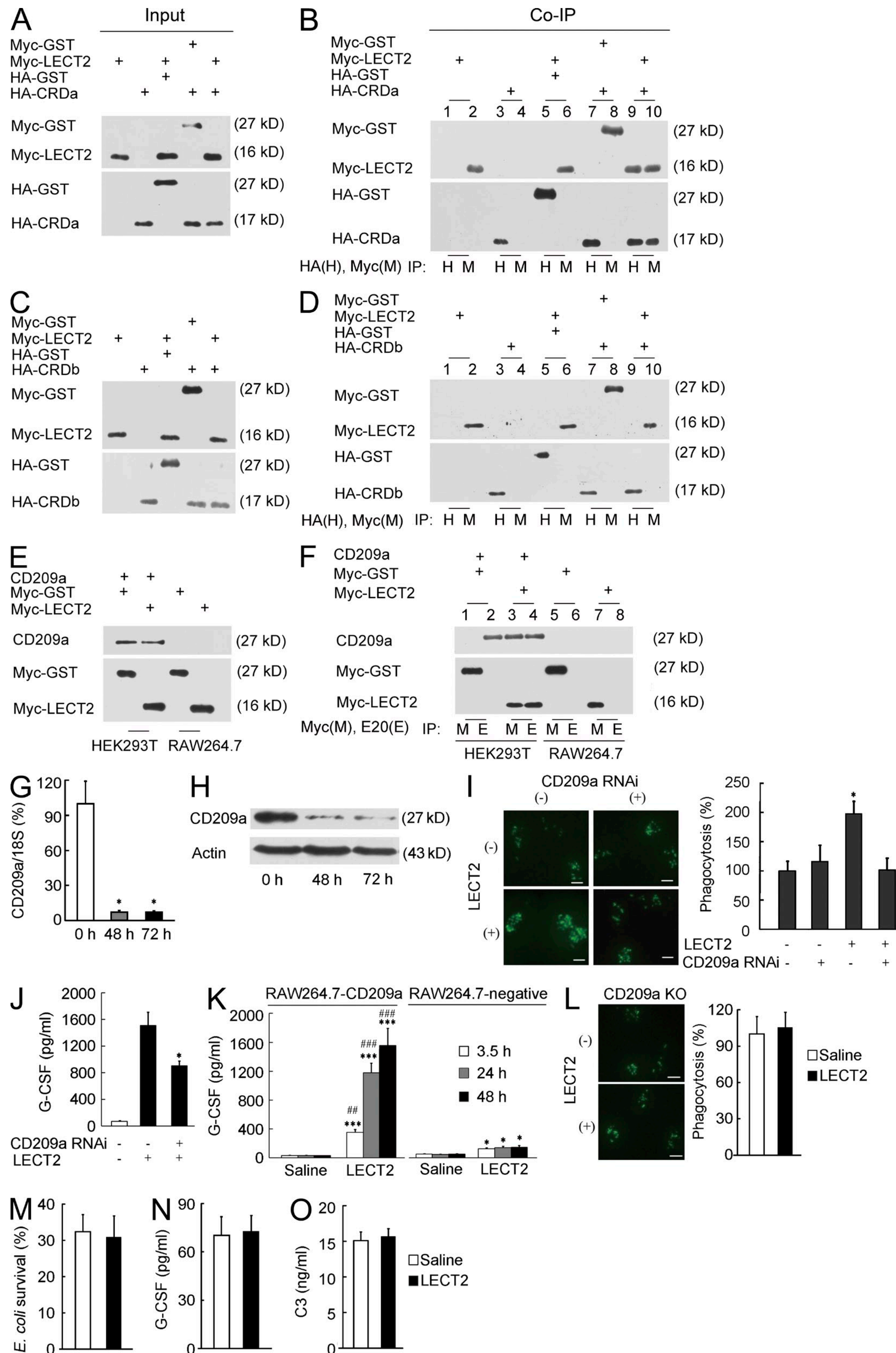

Figure 4. CD209a specifically interacts with LECT2 and mediates the effects of LECT2 on mouse MΦ activation. (A and B) Co-IP experiments were performed with lysates from HEK293T cells expressing Myc-LECT2, HA-CRDa, Myc-LECT2 and HA-GST, HA-CRDa and Myc-GST, and Myc-LECT2 and HA-CRDa, respectively. Co-IP of Myc-LECT2 and HA-CRDa was detected in lanes 9 and 10, whereas corresponding control reactions were negative (lanes 1–8). (C and D) Co-IP experiments were performed with lysates from HEK293T cells expressing Myc-LECT2, HA-CRDb, Myc-LECT2 and HA-GST, HA-CRDb

in HEK293T cells expressing LECT2 and the CRD of CD209a (Fig. 4, A and B). We next evaluated whether the interaction between LECT2 and CD209a was specific. The CRD of CD209b and LECT2 in HEK293T cells did not result in Co-IP (Fig. 4, C and D), indicating that LECT2 specifically interacts with the CRD of CD209a. LECT2 and full-length CD209a were coexpressed in HEK293T cells, and Co-IP of them was observed (Fig. 4, E and F). No Co-IP occurred in RAW264.7 cells expressing LECT2 alone because CD209a was not normally expressed in this cell line (Fig. 4 F).

To further demonstrate whether CD209a mediates LECT2-induced MΦ activation, CD209a expression was knocked down by transfection of peritoneal MΦ with CD209a-specific siRNA (Fig. 4, G and H). The knockdown of CD209a did not alter MΦ phagocytosis of *E. coli*–FITC (Fig. 4 I). In contrast, the lack of CD209a in siRNA-treated MΦ prohibited LECT2 from affecting phagocytosis. G-CSF was used as an indicator of LECT2-mediated protein secretion in MΦ because of its dramatic up-regulation after LECT2 treatment. LECT2-induced G-CSF production in MΦ was abolished in CD209a siRNA-treated MΦ (Fig. 4 J). To specifically study the role of CD209a in mediating the LECT2-treated MΦ activation, another attempt was made to express this receptor in RAW264.7 cells (RAW264.7-CD209a), which do not normally express CD209a, and compare the response to LECT2 in control RAW264.7 cells (RAW264.7-negative). G-CSF secretion was significantly higher in LECT2-treated RAW264.7-CD209a compared with that in RAW264.7-negative (Fig. 4 K). Additionally, LECT2 treatment had no effect on phagocytosis, bacterial killing, G-CSF, and C3 secretion of the MΦ from CD209a-deficient mice (Fig. 4, L–O). Our results indicated that CD209a mediated the effect of LECT2 on MΦ functions, supporting other results showing that CD209 can recognize both self- and nonself-signals (Appelmelk et al., 2003; Tailleux et al., 2003; García-Vallejo and van Kooyk, 2009).

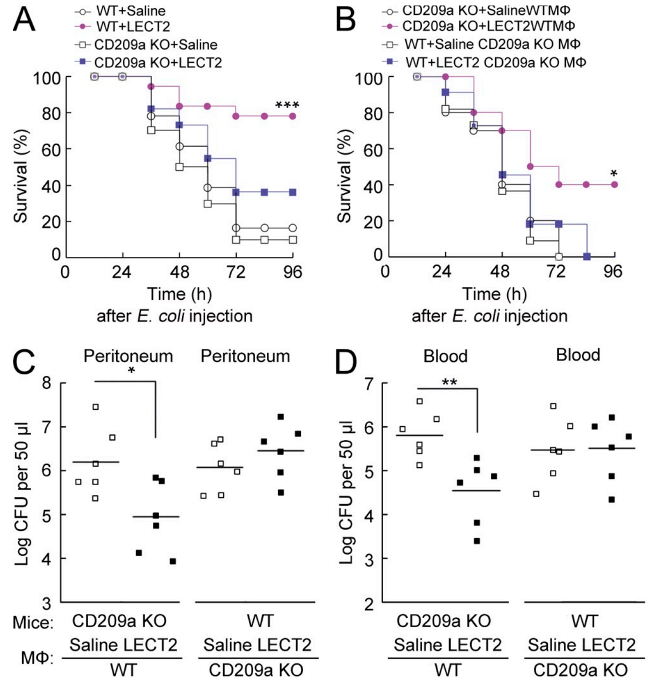

Figure 5. CD209a-expressing MΦ improves survival and reduces bacterial burden in bacterial sepsis. (A) Survival during *E. coli* sepsis in WT and CD209a KO mice treated by LECT2. WT groups, n = 18 per group; CD209a KO/saline, n = 10; CD209a KO/LECT2, n = 11. (B) Survival in WT septic mice received LECT2-treated CD209a KO MΦ (n = 11) or in CD209a KO septic mice received LECT2-treated WT MΦ (n = 10). (C and D) Bacterial burden in the peritoneum (C) or blood (D) of *E. coli*–injected mice received with WT or CD209a KO MΦ (n = 6 per group). Horizontal bars indicate means. Data are representative of two (A and B) or three (C and D) independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

CD209a-expressing MΦ improves sepsis outcome

To confirm whether CD209a mediates protective effect of LECT2 in vivo, the survival was evaluated in CD209a-deficient septic mice treated with LECT2. As shown in Fig. 5 A, LECT2 treatment significantly increased survival in wild-type mice but had no effect on the CD209a-deficient mice. To illustrate whether MΦ activation through the LECT2–CD209a pathway is beneficial to sepsis, we further assessed the survival and bacterial burden of *E. coli* sepsis after adoptive transfer of LECT2-treated MΦ. The survival of CD209a-deficient septic mice receiving LECT2-treated wild-type MΦ was significantly increased compared with those receiving saline-treated wild-type MΦ (Fig. 5 B). Adoptive transfer of LECT2-treated CD209a-deficient MΦ after *E. coli* sepsis had no effect on the survival of wild-type septic mice (Fig. 5 B). Moreover, the bacterial burdens in the peritoneum and blood were reduced in CD209a-deficient septic mice treated with LECT2-treated wild-type MΦ compared with CD209a-deficient septic mice treated with saline-treated wild-type MΦ (Fig. 5, C and D). Adoptive transfer of LECT2-treated CD209a-deficient MΦ had no effect on bacterial burdens in the peritoneum and blood of *E. coli* sepsis

and Myc-GST, and Myc-LECT2 and HA-CRDb, respectively. Co-IP of Myc-LECT2 and HA-CRDb was not detected in lanes 9 and 10. (E and F) HEK293T cells expressing CD209a as well as RAW264.7 cells were manipulated to express Myc-LECT2. Co-IP of CD209a and Myc-LECT2 was detected in HEK293T-Myc-LECT2/CD209a but not in RAW264.7-Myc-LECT2. Representative blots of three independent experiments are shown. (G and H) The knockdown effect after CD209a siRNA transfection was confirmed by RT-qPCR (G) and Western blot (H). Representative blots of three independent experiments are shown. (I) The role of CD209a for LECT2 to enhance phagocytosis was examined. (J) G-CSF secretion of CD209a-knockdown MΦ after LECT2 treatment compared with that of normal MΦ. (K) G-CSF secretion in RAW264.7-CD209a treated with LECT2. (L–O) Effect on the phagocytosis (L), bacterial killing (M), G-CSF (N), and C3 secretion (O) of MΦ from the CD209a-deficient (CD209a KO) mice. Error bars indicate SEM. Data are representative of four (G), seven (I), and at least four (J–O) independent experiments. Bars, 10 µm. *, P < 0.05; ***, P < 0.001. ##, P < 0.01; ###, P < 0.001 versus RAW264.7-negative.

(Fig. 5, C and D). These results confirm that LECT2 signaling in MΦ through CD209a is required for survival in sepsis.

In summary, our study revealed the potential value of clinical application of LECT2 for treatment of sepsis. There is a great deal of controversy as to whether survival in sepsis is dependent on reducing the exaggerated inflammatory response or improving protective immunity (Hotchkiss and Karl, 2003; Kelly-Scumpia et al., 2011). LECT2 activation of MΦ promoted the protective immunity against bacterial infection and improved survival in several relevant sepsis models. Specific interaction between LECT2 and CD209a appeared to be a novel and important mechanism in regulating MΦ activation. Because of its multifunctional roles in various conditions and its effect on MΦ activation, LECT2 is an attractive candidate for further investigation of its effect on treatment in other pathological conditions.

MATERIALS AND METHODS

Mice. Male BALB/c 6–8 wk old mice were purchased from Zhejiang Province Experimental Animal Center. B6 background CD209a knockout mice (B6(FVB)-*Cd209a*$^{tm1.1Cfg}$/Mmucd) were purchased from the Mutant Mouse Resource Regional Center. CD209a$^{+/-}$ mice were intercrossed to generate offspring of all three genotypes, CD209a$^{+/+}$, CD209a$^{+/-}$, and CD209a$^{-/-}$. CD209a$^{-/-}$ mice were used for sepsis and MΦ experiments and their wild-type littermates CD209a$^{+/+}$ were used as the control. The experimental conditions and procedures were approved by the Local Institutional Animal Care and Use Committee and were performed in close agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Production of recombinant mouse LECT2. Recombinant mouse LECT2 proteins were produced from CHO cells as previously described (Yamagoe et al., 1997).

LECT2 detection. The ELISA system was based on one antibody as the capture antibody (rabbit anti-LECT2, C-terminal; Santa Cruz Biotechnology, Inc.) and another antibody for detection (goat anti-LECT2, N-terminal; Santa Cruz Biotechnology, Inc.). For mouse sample collection, a small incision was made on the tail and pressure was applied to the tail to allow collection of ∼50 µl of blood 12 h after *E. coli* or saline injection. Mice recovering from sepsis were subjected to cardiac puncture using a heparinized needle.

Cell culture and chemotactic assay. Blood was collected from mice, and polymorphonuclear leukocytes (PMNs) were isolated using a two-step sedimentation procedure. For peritoneal MΦ, mice were injected i.p. with thioglycollate broth to elicit peritoneal MΦ. Then, MΦ were seeded in culture flasks at 4 × 10^6/ml and rinsed after being allowed to adhere for 2 h at 37°C and 5% CO_2 to remove nonadherent cells. For T and B cell purification, single-cell suspensions of spleen or lymph node were enriched according to the manufacturer's instructions (Miltenyi Biotec). Cell migration assays were performed using a transwell system in a Falcon 24-well culture plate according to the manufacturer's instructions.

To investigate the effect of LECT2 on LPS-stimulated cells, the MΦ were incubated with 5 µg/ml LECT2 in the absence or presence of 100 ng/ml LPS. And then the samples were collected for further experiments at different time points.

Sepsis and bacterial burden determination. For sepsis models with a single bacterial species, bacterial peritonitis was induced by i.p. injection of either 10^8 CFU of live *E. coli* (DH5α) or 5 × 10^6 CFU of live *P. aeruginosa* (ATCC 27853). Survival was monitored once per 12-h period for 96 h. For CLP, mice were anesthetized with 80 mg/kg pentobarbital i.p. The cecum was exposed through a small abdominal midline incision and the cecum below the ileocecal valve was ligated. The ligated part of the cecum was punctured through both surfaces twice with a 22-gauge needle. After repositioning the bowel, the abdomen was closed. Survival was monitored once daily for 8 d. Mice were treated with 0.5 µg/g LECT2 i.v. 30 min after induction of sepsis.

Mice were infected with bacteria and euthanized with i.p. pentobarbital 18 hpi. Blood, livers, spleens, and peritoneal lavage fluids were harvested aseptically. Tissue homogenates, blood, and peritoneal lavage fluid were serially diluted in sterile PBS, plated onto separate Luria-Bertani agar plates, and the plates were incubated for 18 h at 37°C. Colonies were counted separately for each sample.

In vivo PMN and MΦ depletion. PMNs were depleted using an anti–mouse Ly-6G antibody (eBioscience), whereas control animals were treated with nonspecific IgG (eBioscience). The mice were i.v. treated with the antibodies 12 h before *E. coli* injection. MΦ were depleted using clodronate liposomes. The mice were injected i.v. with 0.2 ml of a suspension of clodronate liposomes or PBS 48 h before being infected with *E. coli*. The depletion analysis was performed at 48 h after treatment by flow cytometry (EPICS XL; Beckman Coulter). Ly6G treatment depletes 92% of PMN in blood and 90% of PMN in spleen. Clodronate liposome treatment resulted in an 85% decrease of MΦ in spleen. PMNs were sorted on the basis of expression of Ly6C and CD11b. MΦ were sorted on the basis of expression of F4/80. The PE-conjugated anti-CD11b and FITC-conjugated Ly6C were purchased from BD. The PE-conjugated anti-F4/80 was purchased from eBioscience.

Adoptive transfer of MΦ. Peritoneal MΦ were treated with 5 µg/ml LECT2 or saline for 48 h to activate MΦ. Cell transfer was performed as previously described (Xiong et al., 2009). 10^7 MΦ cells treated with LECT2 or saline suspended in 100 µl pyrogen-free PBS were injected i.v. into the mice.

Measurement of phagocytosis and bacterial killing. *E. coli* strain DH5α cells were labeled with FITC (*E. coli*–FITC). FluoSpheres (F8827) were purchased from Molecular Probes. After incubation with LECT2 for 48 h, FluoSpheres or *E. coli*–FITC was used to measure phagocytosis, which was performed as previously described (Chen et al., 2010). MΦ were incubated with FluoSpheres or *E. coli*–FITC at a multiplicity of infection of 10. The uptake of bacteria or FluoSpheres into cells was captured by a microscope and quantified by measuring fluorescence intensity using ImageJ software (National Institutes of Health). The *E. coli*–FITC uptake was also analyzed by flow cytometry. For C3 and CR3 blockade, MΦ were treated with anti-C3 (diluted 1:100; MP Biomedicals) or anti-CD11b (diluted 1:100; eBioscience) for 30 min before incubation with *E. coli*–FITC. An irrelevant isotype IgG was used as control. Bacterial killing assays were performed as previously described (White et al., 2009). In brief, bacterial survival was determined by dividing the number of colonies in the killing group by those in the uptake group.

Microarray assay. Peritoneal MΦ were treated with 5 µg/ml LECT2 for 3.5 and 48 h, and RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was amplified and labeled using the Low Input Quick Amp Labeling kit (Agilent Technologies). Fluorescence dye-labeled cRNA was then hybridized to the Agilent Mouse Whole Genome 4*44K array. Hybridization, washing, and scanning were performed on a Microarray Platform (Agilent Technologies) according to standard protocols. Raw microarray data were deposited in the Gene Expression Omnibus (GEO) database under accession no. GSE33721.

RT-qPCR. Total RNA was extracted and purified from MΦ using RNAiso reagents (Takara Bio Inc.). After deoxyribonuclease I treatment, cDNA was synthesized using reverse transcription M-MLV (RNase H$^-$; Takara Bio Inc.). Primers were used are as follows (forward and reverse): G-CSF, 5′-GGGAAGGAGATGGGTAAAT-3′ and 5′-GGAAGGGAGACCAGATGC-3′; IFN-γ, 5′-CAACAACATAAGCGTCAT-3′ and 5′-TCAAACTTGGCAATACTC-3′; IL-10, 5′-GGTTGCCAAGCCTTATCGGA-3′ and 5′-ACCTGCTCCACTGCCTTGCT-3′; CXCL-10, 5′-AGTGCTGCCGTCATTTTCTG-3′ and 5′-ATTCTCACTGGCCCGTCAT-3′; IL-1β,

5′-AGAAGCTGTGGCAGCTA-3′ and 5′-TGAGGTGCTGATGTACCA-3′; TNF, 5′-GAACTGGCAGAAGAGGCACT-3′ and 5′-GGTCTGGGC-CATAGAACTGA-3′; CD209a, 5′-CACTGCCTGCCACAATGT-3′ and 5′-CCCAGTACCATGTAGACTCC-3′; and 18S rRNA, 5′-TTTGTTG-GTTTTCGGAACTGA-3′ and 5′-CGTTTATGGTCGGAACTACGA-3′. RT-qPCR was performed using SYBR premix Ex Taq (Perfect Real Time; Takara Bio Inc.). Data were normalized to 18S rRNA.

Cytokine assay. We selected C3, G-CSF, IFN-γ, IL-10, CXCL-10, IL-1β, and TNF for ELISA analysis. The C3 Elisa kit was purchased from Abnova and others were from R&D Systems. The methods were conducted according to the manufacturer's instructions.

Yeast two-hybrid library screening. The yeast Y187 pretransformed mouse peritoneal MΦ cDNA library constructed in pGADT7 (BD) was mated with the AH109 strain transformed with pGBKT7-LECT2, and the library screen was performed as previously described (Antonson et al., 2008).

Co-IP. Antibodies used were: irrelevant mouse IgG1 (BD), anti-Myc, anti-HA (Santa Cruz Biotechnology, Inc.), and anti–CD209a-E20 (GL Biochem) mouse monoclonal antibodies. For Co-IP experiments, 2×10^6 cells were transfected with a total of 20 µg of purified plasmid DNA as indicated. 48 h posttransfection cells were harvested by trypsin digestion and centrifuged, and the cell pellet was lysed in 1 ml lysis buffer for 15 min at 4°C. Cell lysates were precleared for 30 min at 4°C with 40 µl pansorbin (Merck) and 4 µl of non-specific mouse IgG1. After centrifugation at 14,000 rpm for 10 min at 4°C, 200 µl of supernatant was incubated with the indicated antibody (anti–c-Myc, anti-HA, and anti–CD209a-E20) at 4°C overnight. Pansorbin-bound antibodies and proteins were pelleted by centrifugation at 14,000 rpm and washed extensively with PBS on ice, followed by two final washing steps using lysis buffer. Pellets were resuspended in 50 µl of reducing sample buffer, boiled at 95°C for 5 min, and pelleted, and the supernatant was subjected to SDS-PAGE, followed by Western blot analysis.

The eukaryotic expression vector pcDNA3.1 was used in this study. The following transformed cells were used in the experiment: HEK293T cells expressing c-Myc–tagged LECT2 (Myc-LECT2), CD209a CRD-domains fused to an HA tag (HA-CRDa), Myc-LECT2 and HA-GST, Myc-GST and HA-CRDa, Myc-LECT2 and HA-CRDa, CD209b CRD-domains fused to an HA tag (HA-CRDb), Myc-GST and HA-CRDb, Myc-LECT2 and HA-CRDb, and Myc-LECT2 and CD209a (HEK293T-Myc-LECT2/CD209a), respectively. RAW264.7 cells expressing Myc-LECT2 is abbreviated as RAW264.7-Myc-LECT2. Input of expressed and immunoprecipitated Myc-tagged proteins, HA-tagged proteins, and CD209a were visualized by anti-Myc, anti-HA, and anti-CD209a-E20, respectively.

siRNA experiment. Stealth RNAi duplexes against mouse CD209a and a stealth RNAi negative control duplex were purchased from Invitrogen. The sequence of the stealth RNAi duplex used for knockdown of CD209a was as follows: 5′-AUCUACGCCAGCCUUCAACUGGGUC-3′. Transfection of cells with siRNA was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

Transfection. RAW264.7 cells (American Tissue Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS at 37°C. CD209a expression was not detected in RAW264.7 cells. CD209a was cloned into pFBNeo vectors and transfected into HEK293T-based Phoenix ecotropic packaging cells using FuGENE 6, and retroviral supernatants were collected after 48 h for transduction of RAW264.7 cells. Cells were selected and maintained in 0.6 mg/ml geneticin (Sigma-Aldrich) and 0.4 mg/ml Zeocin (Invitrogen). RAW264.7 cells were cultured in 24-well plates at 2×10^6 cells per well. 2 d after transfection, cells were harvested and lysed for analysis.

Western blot. Cell monolayers were washed twice in ice-cold PBS and then scraped in lysis buffer: 20 mM Hepes, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM NaCl, 0.2 mM DTT, 0.5 mM sodium orthovanadate, and 0.4 mM PMSF, pH 7.4. SDS-PAGE, membrane transfer, incubation with antibodies, and ECL reactions were performed. Antibodies were purchased from commercial sources as follows: anti-CD209a and anti-actin (Santa Cruz Biotechnology, Inc.).

Statistical analysis. Results are presented as mean ± SEM. The Kaplan-Meier method was used to analyze survival with SPSS (Version 13.0). Other data were analyzed by one-way ANOVA, followed by the least significant difference post hoc test to compare individual groups. In all cases, $P < 0.05$ was considered statistically significant.

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