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SIGNR1 ligation on murine peritoneal macrophages induces IL-12 production through NFκB activation

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Abstract We have previously shown that murine resident peritoneal macrophages (PEMs) are activated in response to uptake of oligomannose-coated liposomes (OMLs), leading to production of interleukin (IL)-12. To understand the mechanism of activation of PEMs by OMLs, in the present study we investigated the role of a mannose-binding C-type lectin receptor, SIGNR1, in production of proinflammatory cytokines by PEMs, in which SIGNR1 acts as a physiological receptor for OMLs. Engagement of SIGNR1 on PEMs with an anti-SIGNR1-specific rat IgM antibody, ERTR9, induced production of IL-12 and tumor necrosis factor (TNF)-α from PEMs, while secretion of IL-6 and IL-1β was not detected with the same treatment. The level of phosphorylated IκB kinase in PEMs also increased in response to ERTR9 treatment of the cells. Treatment of PEMs with a specific nuclear factor kappa-B (NFκB) inhibitor, BAY11-7082, reduced ERTR9-dependent IL-12 production. Intraperitoneal treatment with BAY11-7082 also led to reduction of subsequent OML-induced IL-12 production from PEMs. These results indicate that SIGNR1-mediated intercellular signaling may induce production of cytokines such as IL-12 through NFκB activation.

Keywords C-type lectin · Interleukin-12 · Macrophage · Nuclear factor kappa-B · SIGNR1

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Abbreviations

APC	antigen-presenting cell
DC-SIGN	dendritic cell-specific ICAM-3-grabbing nonintegrins
OML	oligomannose-coated liposome
PEM	resident peritoneal macrophage
SIGNR1	SIGN-related 1

Introduction

The generation of pathogen-specific immune responses is dependent on the signaling capabilities of pathogen-recognition receptors. Toll-like receptors (TLRs), a family of pattern recognition receptors (PRRs), are key receptors for pathogen recognition [1]. Binding of pathogen-associated molecular patterns (PAMPs) to TLRs triggers signal transduction events that cause activation of mitogen-activated protein (MAP) kinases and transcription factors such as nuclear factor kappa-B (NFκB), leading to pro-inflammatory responses including production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-12, IL-6 and IL-1β. However, signaling from other PRRs is also important in defining pathogen-specific responses. Although antigen-presenting cells (APCs) display a large array of cell surface lectin-like molecules, and these molecules have been shown to act as PRRs [2], the role of these lectin-like receptors in cytokine production is poorly understood.

Molecular structures containing terminal mannose are abundant on the surfaces of many microorganisms and are recognized by mannose receptors expressed on APCs, which are thought to participate in the capture of pathogens [3]. Dendritic cell-specific ICAM-3-grabbing nonintegrins

(DC-SIGN, CD209) expressed on human dendritic cells (DCs) is a well known mannose-binding protein [4, 5] that has been shown to modulate DC maturation by regulating intracellular signaling from TLRs [5]. Pathogens that interact with DC-SIGN are thought to preferentially evoke T helper 2 (Th2)-type or tolerogenic immune responses [6], and engagement of DC-SIGN by specific antibodies has been shown to induce ERK1/2, the activation of which leads to Th2 responses [7].

SIGN-related 1 (SIGNR1, CD209b), a murine homologue of human DC-SIGN [8], is expressed on marginal zone macrophages and resident peritoneal macrophages (PEMs), and mediates capture and internalization of mannose-decorated particles such as viral, bacterial, and fungal pathogens. In contrast to human DC-SIGN, SIGNR1 is thought to act primarily as an endocytotic receptor, since SIGNR1 has no putative signaling motif in its cytoplasmic tail except for a dileucine motif for endocytosis [8]. However, several lines of evidence suggest that SIGNR1 could mediate intercellular signaling [9–11], and that this signaling can result in effector functions that increase pro-inflammatory responses [12].

We have generated liposomes coated with a neoglycolipid constructed from mannotriose and dipalmitoylphosphatidylethanolamine (Man3-DPPE), and shown that SIGNR1 can mediate the specific uptake of the Man3-DPPE-coated liposomes (OMLs) by PEMs isolated from Balb/c mice [13]. In addition, the PEMs produce IL-12 in response to preferential uptake of OMLs, leading to antigen-specific Th1 immunity that is sufficient to reject tumors or parasites in mice [14, 15]. Thus, our observations using OMLs led us to the hypothesis that intercellular signaling mediated by SIGNR1 might participate in the induction of specific immune responses initiated by OML uptake. Recently, we evaluated the signaling ability of SIGNR1 in murine macrophage-like RAW264.7 cells that stably expressed FLAG-tagged SIGNR1 by ligation with an anti-FLAG antibody [16]. Engagement of SIGNR1 molecules in these cells induced activation of MAP kinases and led to TNF-α production [16], indicating that SIGNR1 can transduce signals to induce cytokine production in macrophages.

A rat monoclonal IgM antibody, ERTR9, has been shown to interact strongly with SIGNR1 on PEMs [9]. To understand the specific production of proinflammatory cytokines from PEMs in response to OML uptake, the present study was conducted to investigate whether ligation of SIGNR1 with ERTR9 had functional effects in PEMs. We found that ERTR9 treatment of PEMs induces transient phosphorylation of IκB kinase (IKK) and production of IL-12 and TNF-α, but did not induce production of IL-6 and IL-1β.

Materials and methods

Reagents and antibodies

Unless otherwise indicated, the reagents and antibodies used in this study are the same as those described in previous papers [13, 14]. Biotin-labeled monoclonal antibodies (mAbs) directed against SIGNR1 (ERTR9, rat IgM) and control antibodies were obtained from BMA Biomedicals (Augst, Switzerland) and R&D Systems (Minneapolis, MN, USA), respectively. FITC-labeled streptavidin were purchased from Sigma-Aldrich. Liposomes consisting of DPPC, cholesterol, and Man3-DPPE at a molar ratio of 10:10:1 (OMLs) were prepared as described previously [17].

Isolation and activation of resident peritoneal macrophages (PEMs)

Six- to 8-week-old female Balb/c mice or ICR mice were purchased from Japan SLC (Hamamatsu, Japan) and used 2 days after arrival at the animal facility at Tokai University. A culture dish with a surface grafted with a temperature-responsive polymer (UpCell®, CellSeed Inc. Tokyo, Japan) was used to recover adherent cells [14]. Cells obtained from the peritoneal cavity of the mice were seeded onto an UpCell culture dish and incubated at 37°C for 1 h. After non-adherent cells were removed by extensive washing with pre-warmed culture medium, adherent cells were detached from the dishes by culturing the cells at 20°C for 30 min. Recovered cells were stained with antibodies against various markers after Fc block treatment and analyzed by FACS [13, 18]. Over 90% of these cells stained positively with anti-CD11b [18].

Ligation of SIGNR1 on PEMs

In all experiments, 2×10^6 PEMs were used for each condition. The cells were washed extensively and cultured in serum-free DMEM for 18 h to reduce the basal level of activation, and the resulting cells were suspended in ice-cold serum-free DMEM and 5 μg/ml of ERTR9 (an anti-SIGNR1 monoclonal antibody) was added to the cell suspension. As positive and negative controls, LPS (1 μg/ml) and rat IgM (5 μg/ml), respectively, were added to the suspension. The mixture was transferred to a 37°C water bath and incubated for the indicated time. To analyze the signaling pathway, dimethylsulfoxide (DMSO) solutions of a specific inhibitor of NFκB (BAY11-7082; 10 μM) were added 30 min prior to stimulation with the antibody. For assessment of cytokine production, PEMs were treated with antibodies and incubated overnight at 37°C, and then the levels of IL-1β, IL-6, IL-12 p40, and TNF-α secreted in the culture supernatants were quantified using commercial ELISA kits (Becton Dickinson).

To analyze activation of intracellular signaling molecules, cells were immediately lysed with 200 μl of a solution containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 200 mM Na_3VO_4, 0.5% Brij 58, and protein inhibitor cocktail (Sigma-Aldrich, St Louis, MO) after stimulation of cells with ERTR9, LPS or CpG. The cell lysates were centrifuged at 3,000 rpm for 10 min and the supernatants were collected and stored at -30°C until assayed. An aliquot of each supernatant was used to determine the protein concentration using a modified Lowry protein assay reagent (Pierce, Rockford, IL). The remaining sample was subjected to SDS-PAGE on 4–20% gradient gels under reducing conditions and transferred onto a PVDF membrane. After blocking with 3% BSA in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20, the membranes were incubated with primary antibodies specific for the activated/phosphorylated forms of various signaling molecules and then with horseradish peroxidase-conjugated secondary antibodies. Detection of phospho-IKK and phospho-MAP kinases was carried out using a rabbit polyclonal anti-phospho-IKKα/β antibody (Cell Signaling, Beverly, MA), a rabbit polyclonal anti-phospho-ERK1/2 antibody (Cell Signaling), a rabbit polyclonal anti-phospho-p38 MAPK antibody (Cell Signaling), and a rabbit polyclonal anti-phospho-JNK antibody (Cell Signaling). As a control for protein loading, membranes were probed with rabbit polyclonal antibodies against α-tubulin (Santa Cruz Biotechnology).

Inhibition of NFκB in PEMs in the peritoneal cavity

BAY11-7082 dissolved in PBS (100 μl) containing 1% DMSO (or 100 μl of PBS containing 1% DMSO as a negative control) was injected into the peritoneal cavity and OMLs (60 μg of cholesterol) with entrapped FITC-BSA were administered 30 min later. Peritoneal cells were harvested 15 min after administration of OMLs and some of these cells were used for evaluation of OML uptake. Briefly, peritoneal cells were washed twice with PBS, treated with Fc block, and then stained with a PE-labeled anti-CD11b mAb to detect PEMs. Fluorescent signals from FITC in CD11b-positive cells were analyzed to show specific uptake of OMLs in PEMs. PEMs were separated from the peritoneal cells and cultured in a 12-well plate for 24 h, after which IL-12 production was measured in the culture media.

Results

Ligation of SIGNR1 on PEMs by ERTR9 induces production of IL-12 but not of IL-6

We have shown previously that SIGNR1 can mediate intraperitoneal uptake of OMLs by PEMs of Balb/c mice [13]. To analyze whether SIGNR1 is involved in cytokine production from PEMs in response to OMLs, we assessed the effect of engagement of SIGNR1 by ERTR9, a specific IgM antibody against SIGNR1. Most PEMs isolated from Balb/c mice strongly reacted with ERTR9 (Fig. 1a), indicating that about 80% of PEMs strongly express SIGNR1 on their surfaces. To engage SIGNR1 on PEMs, ERTR9 (5 μg/ml) was added to cultures of PEMs. The cells were then incubated for 24 h and the levels of proinflammatory cytokines secreted in the culture medium were determined by ELISA. For positive and negative controls, the cells were treated with LPS (1 μg/ml) and rat IgM (5 μg/ml), respectively. As shown in Fig. 1, LPS treatment led to production of significant levels of IL-1β and IL-6 from PEMs, while the cells treated with ERTR9 did not produce these proinflammatory cytokines. In contrast, PEMs produced significant levels of IL-12 and TNF-α when treated with ERTR9 (Fig. 1d,e). Control rat IgM did not induce production of these cytokines. Secretion of IL-12 from ERTR9-treated cells occurred in an antibody concentration-dependent manner (Fig. 1f). Secretion of IL-6 or IL-1β was not observed even at high antibody concentrations (data not shown).

SIGNR1 engagement leads to phosphorylation of IκB kinase

Expression of proinflammatory cytokines is regulated by the transcription factor NFκB, which translocates to the nucleus and activates transcription of multiple genes encoding cytokines, including IL-12, TNF-α, and IL-6. Activation of IKK leads to specific IκBα phosphorylation and degradation, with subsequent release of NFκB, and is controlled by serine phosphorylation in the activation loop of the kinase domain [19, 20]. Therefore, we investigated phosphorylation of IKK in PEMs by engagement of SIGNR1 with 5 μg/ml of ERTR9. LPS is known to trigger intracellular signaling mediated by TLR4, leading to activation of NFκB. As shown in Fig. 2a, the level of IKK phosphorylation increased significantly (by about 3-fold) 10 min after PEMs were stimulated with LPS. The level of phospho-IKK in PEMs was also clearly enhanced (by about 2-fold) within 10 min after treatment with ERTR9. The level of phospho-IKK in LPS-activated PEMs remained higher than that in non-stimulated PEMs for at least 60 min after activation, whereas ERTR9-induced IKK phosphorylation was a transient event that reached a peak at 20 min after treatment and vanished after 60 min. Phosphorylation of ERK1, JNK, and p38 also increased within 10 min after cells treated with ERTR9, and ERK2 phophorylation started 20 min after the treatment (Fig. 2b). These results suggested that MAPK pathways are also activated by ligation of SIGNR1.

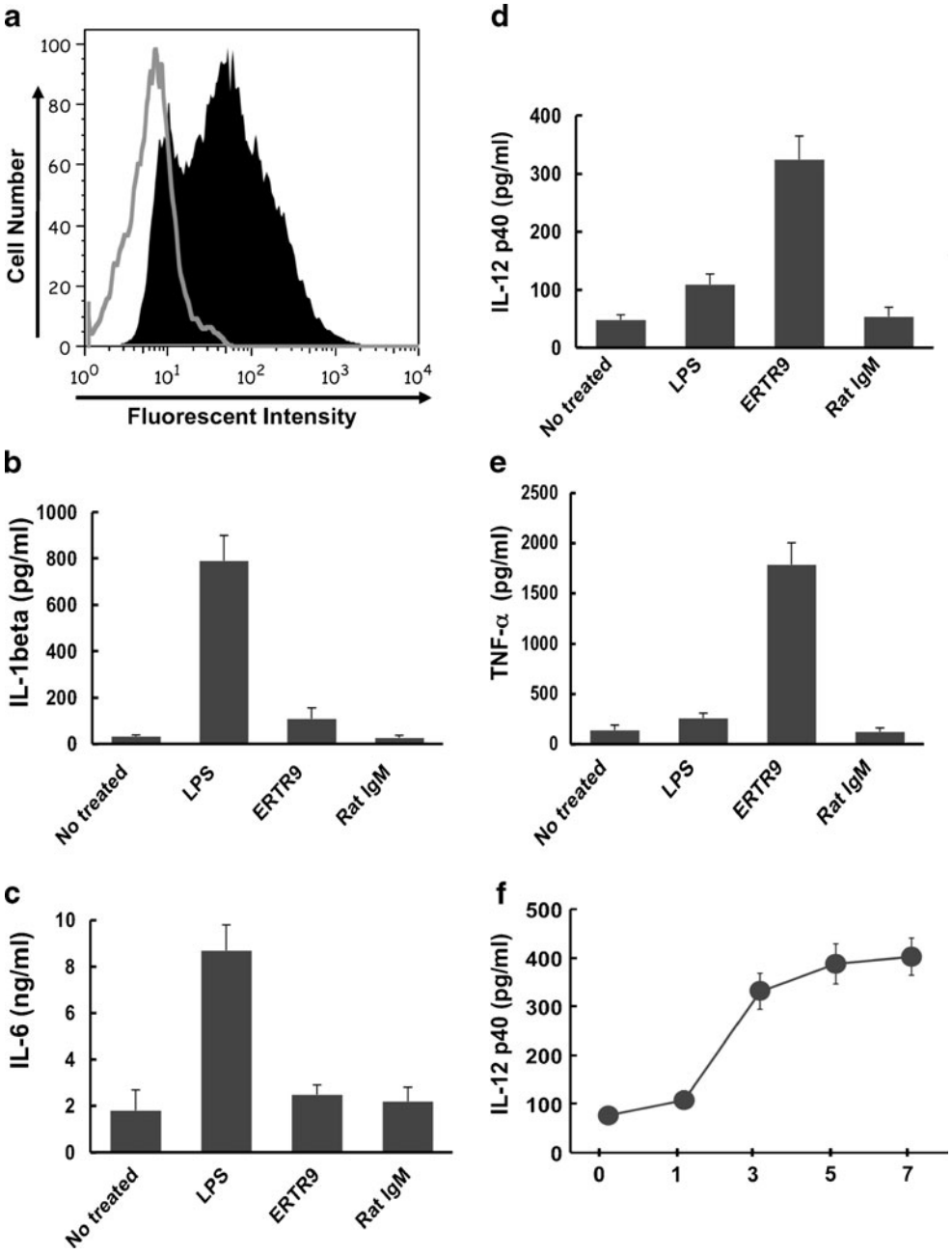

Fig. 1 Cytokine production from PEMs induced by antibody treatment. **a**. PEMs isolated from Balb/c mice were stained with ERTR9 (shaded peak) or type control (open peak). **b-e**. PEMs (2×10^6 /ml) were treated with ERTR9 (5 μg/ml), LPS (1 μg/ml) or rat IgM (5 μg/ml) in serum-free medium for 24 h, and the IL-1β (**b**), IL-6 (**c**), IL-12 p40 (**d**), and TNF-α (**e**) levels secreted in the culture medium were determined by ELISA. F. PEMs were treated with different concentrations of ERTR9 and the level of IL-12 p40 secreted in the culture medium was determined. Each bar represents the mean ± SD of four independent experiments

Effects of an inhibitor of NFκB on ERTR9-induced cytokine production from PEMs

To assess whether NFκB is involved in cytokine production in response to ERTR9 treatment, PEMs were pretreated with Bay11-7082, an inhibitor of NFκB activation, and cytokine production from the cells was determined (Fig. 3). Inhibition of NFκB activation with Bay11-7082 almost completely abolished LPS-induced production of IL-12 p40 from PEMs. Similarly, ERTR9-induced production of IL-12 p40 was significantly but not completely blocked by treatment of the cells with Bay11-7082.

Effects of an NFκB inhibitor on OML-induced IL-12 production from PEMs

Intraperitoneal administration of OMLs leads to IL-12 production from PEMs and SIGNR1 can mediate specific uptake of the OMLs by PEMs *in vivo* [13, 18]. To investigate whether the NF-κB pathway participates in OML-induced IL-12 production from PEMs, as for ERTR9-induced IL-12 production, Bay11-7082 was injected into the peritoneal cavity before OML administration and PEMs were recovered from the cavity 30 min after OML administration. IL-12 production from PEMs induced by OMLs was clearly inhibited by injection of 50 nmol of

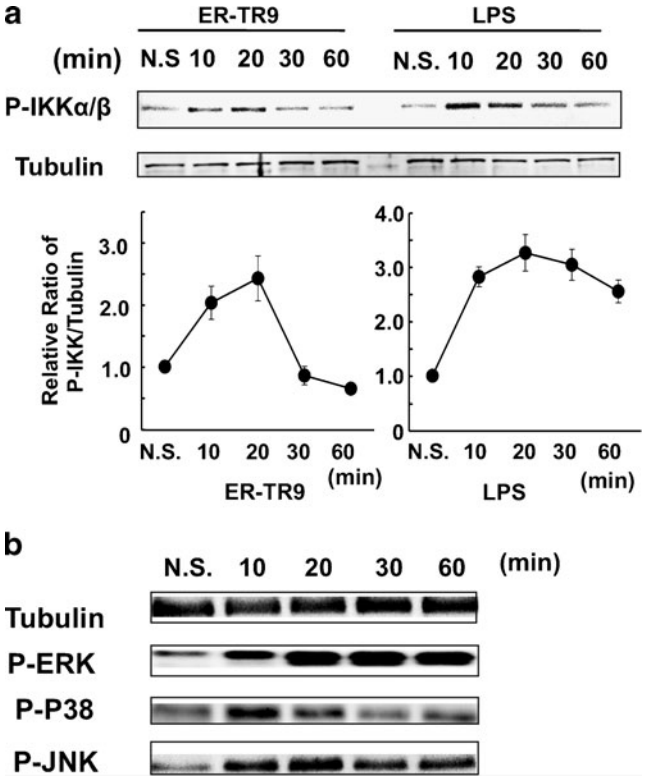

Fig. 2 Activation of IKK by ERTR9 in PEMs. PEMs were cultured in serum-free DMEM for 18 h to reduce the basal level of activation, and the resulting cells were treated with ERTR9 (5 μg/ml) or LPS (1 μg/ml) at 37°C for the indicated time. After stimulation, cells were immediately lysed and the lysates were subjected to SDS-PAGE on 4-20% gradient gels under reducing conditions and analyzed by immunoblotting for phospho-IKK (p-IKK) (**a**) or phospho-MAP kinases (**b**). Tubulin was used as a control for protein loading in the lanes. The relative ratios of p-IKK/tubulin are shown in panel A. Each bar represents the mean ± SD of three independent experiments. N.S. indicates no stimulation of cells

Bay11-7082, although OML uptake by PEMs was not affected by the inhibitor (Fig. 4a,b). Inhibition of OML-induced IL-12 production by Bay11-7082 occurred in a concentration-dependent manner (Fig. 4c).

Discussion

We have shown previously that liposomes coated with a neoglycolipid consisting of mannotriose and dipalmitoyl-phosphatidylcholine (OMLs) are preferentially ingested into PEMs in mice. The PEMs mature with upregulation of MHC class II and costimulatory molecules and produce IL-12 in response to intraperitoneal uptake of OMLs [18]. In addition, OMLs are recognized and captured by SIGNR1 expressed on PEMs, and we have shown that SIGNR1 acts as a receptor for OMLs on PEMs *in vivo* [13]. Thus, the aim of the current study was to define whether SIGNR1 was associated with OML-induced IL-12 production from PEMs.

In this study, we showed that treatment of PEMs with ERTR9, a SIGNR1-specific antibody, induced production of the proinflammatory cytokines IL-12 and TNF-α from PEMs. Since treatment with a control rat IgM did not induce production of these cytokines, this effect might be directed by ERTR9 ligation of SIGNR1 on the cells. We have shown that crosslinking of SIGNR1 molecules with an anti-FLAG antibody on murine macrophage-like RAW264.7 cells that stably expressed FLAG-tagged SIGNR1 leads to TNF-α production [16]. Therefore, TNF-α production from PEMs in response to ERTR9 treatment is consistent with our previous results. In addition to TNF-α, antibody ligation of SIGNR1 on PEMs led to IL-12 production. Interestingly, ERTR9-treated cells did not produce other proinflammatory cytokines such as IL-1β and IL-6, although LPS-stimulated PEMs produced significant levels of IL-1β and IL-6. Since PEMs with ingested OMLs produce significant levels of IL-12, but production of IL-1β and IL-6 from these cells is suppressed in a dose-dependent manner [18], the profile of cytokine production from PEMs treated by ERTR9 was consistent with that from PEMs with ingested OMLs.

We also found that ERTR9 treatment of PEMs led to transient phosphorylation of IKKs. Activation of IKK has been demonstrated to be controlled by phosphorylation [21] and the activated IKK complex phosphorylates IκB, resulting in nuclear translocation of NFκB, which induces expression of proinflammatory cytokines such as IL-12 [1]. Therefore, the increased levels of phosphorylated IKKs in ERTR9-treated PEMs suggest the involvement of NFκB in SIGNR1-mediated cytokine production, as for TLR-mediated proinflammatory cytokines. This may be supported by the finding that PEMs treated with Bay11-7082, an inhibitor of NFκB activation, showed significantly

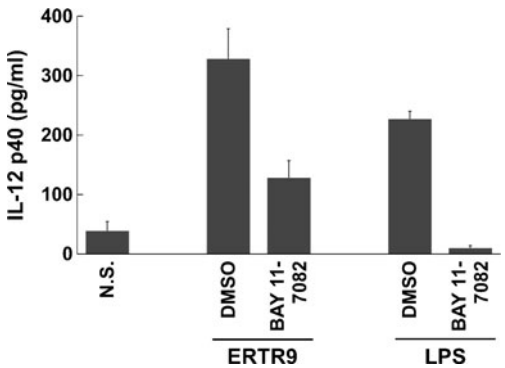

Fig. 3 Effects of Bay11-7082 on production of IL-12 from PEMs. Fresh medium containing an inhibitor of NFκB activation (Bay11-7082, 10 μM) was added to PEM cultures 30 min prior to addition of 5 μg/ml of ERTR9 or 1 μg/ml of LPS. Control cells were treated with medium containing 0.1% DMSO. The cells were incubated overnight at 37°C and the IL-12 level in the culture supernatant was analyzed by ELISA. Each bar represents the mean ± SD for three experiments. N.S. indicates no stimulation of cells

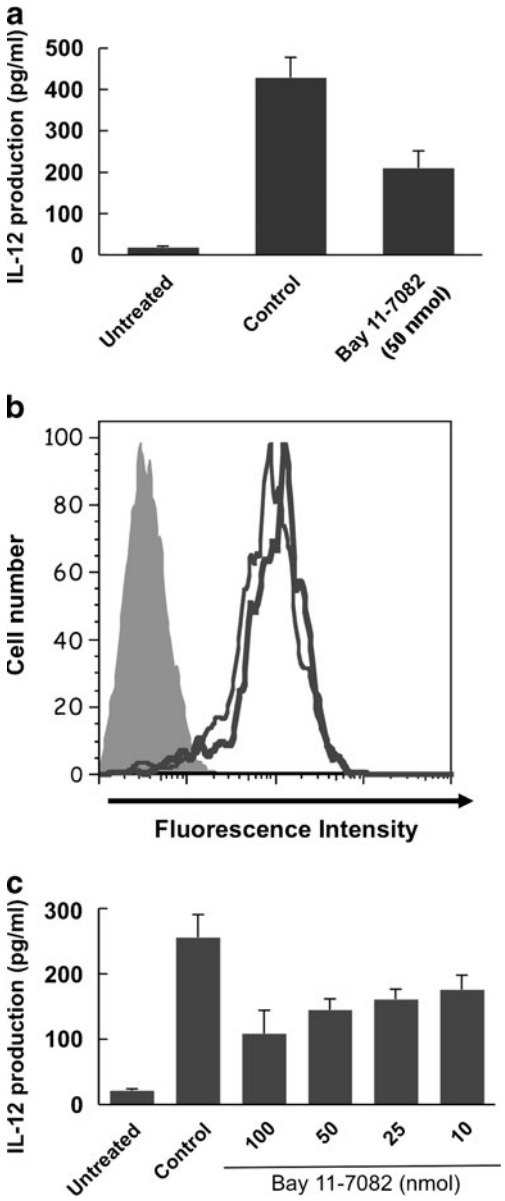

Fig. 4 Effects of *in vivo* treatment with an inhibitor of NFκB activation on production of IL-12 from PEMs in response to OML uptake. (**a**) BAY11-7082 (50 nmol) dissolved in PBS containing 1% DMSO was injected into the peritoneal cavity of Balb/c mice and then OMLs (60 μg of cholesterol) with encased FITC-BSA were administered. As a control, 100 μl of PBS containing 1% DMSO was injected before administration of the OMLs. PEMs were recovered from the peritoneal cavity and cultured for 24 h in a 24-well plate at a cell density of 10^6 /ml, and then the IL-12 level in the medium was determined. Each bar represents the mean ± SD for four experiments. (**b**) Some PEMs were used for evaluation of OML uptake in the peritoneal cavity. Thin and thick lines indicate fluorescent signals from FITC in $CD11b^+$ cells obtained from BAY11-7082- and PBS-treated mice, respectively. The shaded peak indicates PEMs from mice that were not administered OMLs. (**c**) Before administration of OMLs, different concentrations of BAY11-7082 were injected into the peritoneal cavity of mice. PEMs were then recovered from peritoneal cavity and cultured for 24 h, and the IL-12 level in the culture medium was determined

reduced ERTR9-induced production of IL-12. We also found that OML-induced IL-12 production was inhibited *in vivo* by Bay11-7082. Since SIGNR1 is a physiological receptor for OMLs on PEMs *in vivo* [13], SIGNR1 ligation by OMLs *in vivo* may induce specific cytokines, including IL-12, through NFκB activation.

It is unclear how SIGNR1 ligation leads to activation of the NF-κB pathway. It may be possible that TLR4-mediated signal transduction triggers by SIGNR1 ligation by ERTR9 as well as OMLs, since SIGNR1 has been shown to functionally associate with TLR4 to capture gram-negative bacteria and enhance TLR4-mediated responses [11]. However, we showed in this paper that LPS treatment leads to significant production of IL-1β and IL-6 from PEMs, whereas negligible amounts of these cytokines and significant amounts of IL-12 are produced from PEMs by the treatment with ERTR9 (Fig. 1). Therefore, signaling pathway triggered by SIGNR1-ligation might differ from that by TLR4. On the other hand, it has been also shown that signaling triggered by dectin-1, a C-type lectin receptor for β-glucan, combines with TLR2-TLR6 signaling to enhance production of specific cytokines such as IL-12 [22]. We have recently demonstrated that SIGNR1 and CR3 cooperate to recognize OMLs *in vivo* [13, 23]. Thus, it may also be possible that cross-talk between signaling mediated by SIGNR1 and that by other receptors results in generation of specific cytokine profiles induced by OMLs in PEMs.

ERTR9-induced IL-12 production was not completely abolished by Bay11-7082, while LPS-induced cytokine production was almost completely inhibited by the same concentration of Bay11-7082 *in vitro*. Bay11-7082 is a selective inhibitor of phosphorylation of IκB, which is regulated by IKKs, and this leads to decreased nuclear translocation of NF-κB [24]. Interferon regulatory factors (IRFs) regulate genes in the innate immune response, and IRF-3 is activated through phosphorylation by IKK or TBK1 [25]. IKKα was recently shown to phosphorylate IRF-7 in the TLR-MyD88-TRAF6 pathway and to activate type 1 interferon production [26]. In addition, IKKα phosphorylates IRF-5 and induces formation of IRF-5 dimers [27]. Therefore, IKK-associated mechanisms other than NFκB activation might also contribute to preferential cytokine production in ERTR9-treated PEMs.

In conclusion, intracellular signaling mediated by a mannose-binding C-type lectin receptor, SIGNR1, may control preferential production of cytokines from PEMs through activation of the NFκB pathway. Since we have demonstrated that SIGNR1 acts as a physiological receptor for OMLs on the surface of PEMs, our results may provide a molecular explanation for the ability of OMLs to induce production of cytokines from PEMs. The results suggest that a novel signaling pathway mediated by SIGNR1

independent of TLR-mediated signaling might exist in PEMs, and the precise molecular mechanisms are currently being investigated.

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