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Late Endosome/Lysosome-Localized Rab7b Suppresses TLR9-Initiated Proinflammatory Cytokine and Type I IFN Production in Macrophages

Ming Yao,2*† Xingguang Liu,2† Dong Li,* Taoyong Chen,† Zhen Cai,* and Xuetao Cao3*†

Inappropriate activation of TLR9 has been found to be involved in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus. TLR9 antagonists have been proposed to be therapeutic for some kinds of autoimmune diseases. In contrast, new negative regulators of TLR9 signal pathway need to be identified, and the mechanisms for the control of TLR9 response need to be fully investigated. It is well known that TLR9 will be finally transported to late endosome/lysosome once activated; however, the exact mechanism and the biological significance of the redistribution have not been fully elucidated. Ras related in brain (Rab)7b is a small guanosine triphosphatase, identified by us before, which is mainly localized in late endosome/lysosome. Our previous study shows that Rab7b can negatively regulate TLR4 signaling by promoting lysosomal degradation of TLR4. In this study, we show that TLR9 ligation can inhibit Rab7b expression in macrophages via ERK and p38 activation. In turn, the late endosome/lysosome-localized Rab7b can colocalize with TLR9 in lysosomal-associated membrane protein 1-positive compartment and down-regulate the expression of the TLR9 in macrophages by promoting TLR9 degradation once TLR9 is activated. Accordingly, Rab7b can negatively regulate TLR9-triggered production of TNF-α, IL-6, and IFN-β in macrophages by impairing activation of MAPKs and NF-κB pathways. Our results suggest that the late endosome/lysosome-localized Rab7b can down-regulate TLR9-triggered proinflammatory cytokine and type I IFN production by impairing TLR9 signaling via promotion of TLR9 degradation. The Journal of Immunology, 2009, 183: 1751–1758.
Ras related in brain (Rab) proteins are members of the Ras-like small guanosine triphosphatase (GTPase) superfamily of proteins and constitute the largest branch of this family, with over 60 members in human and 11 in the budding yeast. One remarkable characteristic of Rab family proteins is their specific localizations within certain subcellular organelle (15). The known function for Rab family proteins is the regulation of myriad trafficking processes, including vesicle formation, motility, tethering, and fusion processes (16, 17). However, more and more evidence shows that Rab proteins also participate in other biological processes. For instance, the late endosome-localized small GTPase Rab7 has been reported to participate in the growth factor-regulated cell nutrition and apoptosis by mediating the internalization and degradation of nutrient transporters (18). Recently, Rab25 has been found to be related to the pathogenesis of breast cancer and ovarian cancer. In addition, up-regulation of Rab5a and Rab7 occurs in thyroid-associated adenomas (19). This evidence proves that Rab family proteins are involved in many other biological processes more than their known functions. However, whether and how Rab family proteins regulate TLRs response remains to be clarified.

Protein degradation is one of the effective ways for the negative regulation of membrane receptors. We previously identified a novel small GTPase, homologous to Rab7, which is named as Rab7b. Rab7b is localized to late endosome/lysosome-associated compartments and selectively expressed in monocytic cells (20). We demonstrated that Rab7b could negatively regulate TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4 (21). TLR4 is the cell membrane-localized TLR; however, TLR9 is an intracellular-localized TLR that will traffic to endolysosomes from the endoplasmic reticulum (ER) upon ligand-induced stimulation (22). These two kinds of TLRs represent different signal initiations of TLRs from cytoplasmic membrane and intracellular compartment, respectively. Therefore, we wondered whether the late endosome/lysosome-localized Rab7b could affect the trafficking of intracellular localized TLR9 and regulate TLR9 signaling in macrophages. In this study, we demonstrate that Rab7b functions as a negative regulator of TLR9 signaling in macrophages by enhancing trafficking of TLR9 to the endosome/lysosome and promoting degradation of TLR9, thus leading to the suppression of TLR9-triggered production of proinflammatory cytokines such as TNF-α, IL-6, and IFN-β.

Materials and Methods

Mice and reagents

C57BL/6 mice (5–6 wk old) were purchased from SIPPR-BK Experimental Animal. All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai. Phosphorothioate-modified CpG-oligodeoxynucleotide (ODN) was synthesized by Shenggong, and its sequences are as follows: 5'-TCC ATG ACC TTC CTG ATG CT-3'. Endotoxin in these ODNs was removed by endotoxin removal solution from Sigma-Aldrich, and endotoxin level was less than 0.015 endotoxin U/mg. Rab7b stably overexpressed or silenced RAW264.7 cell clones were selected in 600 g/ml G418 for 3–4 wk and then confirmed by Western blot for the expression of Rab7b.

Construction of expression plasmid

The HA-tagged recombinant vector-encoding mouse Rab7b was constructed by PCR-based amplification, and then subcloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen). Tags were placed at the N terminus of Rab7b cDNA to avoid interfering with Rab7b localization. The clone was confirmed by DNA sequencing.

RT-PCR and quantitative PCR

Total cellular RNA was extracted using TRIzol reagent (Invitrogen). A quantity amounting to 2 μg of total RNA was used in a 20 μl reverse-transcription reaction using the First Strand cDNA Synthesis kit (Toyobo), and then the cDNA was diluted into 160 μl as the template of the next quantitative PCR. Quantitative PCR was performed on a MUR Chrono4 Continuous Fluorescence detector (Bio-Rad), according to the manufacturer’s protocol and as described.

RNA interference assay

For transient transfection, Rab7b small interfering RNA (siRNA) was synthesized as follows: 5’-UGGUACCAACAUUCUACAATT-3’ (Rab7b

![FIGURE 1. Activation of TLR9 inhibits Rab7b expression via ERK pathway. A. Primary peritoneal macrophages were treated with 0.3 μM CpG-ODN for 2, 4, 8, 12, and 24 h, respectively. mRNA expression level of Rab7b was assayed by quantitative PCR. The results were presented as fold expression of Rab7b mRNA to that of β-actin. B. RAW264.7 cells were pretreated with 10 μM PD98059, 10 μM SP600125, 5 μM SB203580, 100 μM pyrroline dithiocarbamate, and DMSO as negative control for 30 min, and then stimulated with 0.3 μM CpG-ODN for 2, 4, 8, 12, and 24 h. mRNA expression level of Rab7b was determined by quantitative PCR. Similar results were obtained in three independent experiments. Data are shown as mean ± SD of three independent experiments. **, p < 0.01.

pared and cultured, as described previously (23). Rab7b stably overexpressed or silenced RAW264.7 cell clones were selected in 600 μg/ml G418 for 3–4 wk and then confirmed by Western blot for the expression of Rab7b.
siRNA 216, targeting at the site of 216) and 5'-H11032-UUGUAGAAUGUU
GAUACCATT-3'. Rab7b siRNA 427, targeting at the site of 427). The
control small RNA sequence was 5'-H11032-AATCAGTCACGTTAATGGTCG-
3'. siRNA duplexes were transfected into RAW264.7 cells or mouse peri-
toneal macrophages (5 × 10⁵) were transfected with HA-Rab7b or mock vector. For
ty-eight hours later, the cells were stimulated with 0.3 μM CpG-ODN for the indicated time.
Relative mRNA expression of TNF-α, IL-6, IFN-α, and IFN-β was measured by quantitative PCR. B,
Mouse primary peritoneal macrophages (5 × 10⁵) were transfected with HA-Rab7b or mock vector.
Measurement of cytokine production
Peritoneal macrophages (5 × 10⁵) or RAW264.7 cells (2 × 10⁵) were
seeded in 24-well plates and cultured overnight. The cells were transfected with indicated amounts of Rab7b expression plasmid, followed by the man-
facturer’s protocols, and cultured at least for 36 h. After stimulation with 0.3 μM CpG-ODN for indicated time, the supernatants were harvested.
Western blotting
A total of 1 × 10⁶ RAW264.7 cells with Rab7b stable overexpression and
mock control RAW264.7 cells was seeded in 6-well plates and cultured
overnight, respectively. After 0.3 μM CpG-ODN stimulation, cells were
washed twice with cold PBS, and lysed with 1× cell lysis buffer (Cell
Signaling Technology) containing protease inhibitor mixture (Calbio-
chem). Equal amounts of protein were subjected to SDS-PAGE and trans-
ferred onto nitrocellulose membranes, and immunoblot was performed, as
described previously (23).
Statistical analysis
All experiments were independently performed three times in triplicate. Results are given as means plus or minus the SE or SD. Comparisons
between two groups were performed using Student’s t test, whereas com-
parisons between multiple groups were done using AVONA test, with a
value of p less than 0.05 considered to be statistically significant.
Assay of luciferase reporter gene expression
HEK293 cells were cotransfected with the mixture of indicated lucif-
erase reporter plasmid, pRL-TK-Renilla-luciferase plasmid, and indi-
cated amounts of Rab7b, MyD88, or TLR9 construct. Total amounts of
plasmid DNA were equalized with empty control vector. After 24 h, the
cells were left untreated or treated with 0.3 μM CpG-ODN. Luciferase
activities were measured using Dual-Luciferase Reporter Assay System
(Promega), according to the manufacturer’s instructions. Data are nor-
malized for transfection efficiency by dividing Firefly luciferase activity
with that of Renilla luciferase (25).
Immunofluorescence staining and confocal microscopy
The cells with Rab7b stable overexpression were cultured on coverslips
overnight. After CpG-ODN stimulation, cells were washed with PBS twice
before being fixed with 4% paraformaldehyde in PBS for 15 min at room
temperature, and then permeabilized and blocked with PBS containing 1
mg/ml saponin and 5 mg/ml BSA for 1.5 h. Staining with primary Abs was
performed for 1 h at room temperature in blocking buffer. After washing,
samples were incubated with appropriate secondary Abs (1:200). Slides
were finally examined by a Leica TCS SP2 confocal laser microscopy
(Leica Microsystems) under a ×20 objective lens and analyzed using Leica
Confocal Software.
Results
Ligation of TLR9 inhibits Rab7b expression in macrophages via ERK and p38 pathway

First, we investigated whether the expression of Rab7b in macrophages was affected after activation of TLR9. RAW264.7 and mouse primary peritoneal macrophages were treated with 0.3 μM CpG-ODN for indicated time. We found that mRNA expression of Rab7b decreased dramatically 2 h after CpG-ODN stimulation and continued to be decreased until 8 h in primary peritoneal macrophages. Then mRNA expression of Rab7b slightly increased later, but still less than the normal level (Fig. 1A). The similar pattern of Rab7b mRNA expression decrease was also observed in RAW264.7 cells after CpG-ODN stimulation (Fig. S1A). The data showed that TLR9 activation will lead to the down-regulation of Rab7b expression in macrophages.

Ligation of TLR9 will finally activate ERK, p38, JNK, and NF-κB pathways in cascade (2). Next, we explored which signaling pathway was responsible for the inhibition of Rab7b expression once TLR9 activated. As shown in Fig. 1B, the reduction of the Rab7b mRNA expression in TLR9-triggered macrophages was reversed by ERK inhibitor and p38 inhibitor, but not NF-κB or JNK inhibitors. Furthermore, we stimulated primary peritoneal macrophages from wild-type and TLR9−/− mice to assay mRNA expression of Rab7b. Rab7b mRNA expression in the resting TLR9−/− macrophages was higher than that in wild-type macrophages. Moreover, the dynamic changes of Rab7b mRNA expression during CpG-ODN treatment disappeared in TLR9−/− macrophages compared with wild-type cells (Fig. S1B). Thus, knockout of TLR9 and impairing its signaling indeed led to markedly increased expression of Rab7b mRNA. These results suggest that the activation of TLR9 signaling inhibits the transcription of Rab7b, which is mainly dependent on the activation of ERK and p38 pathway.

Rab7b down-regulates TLR9-triggered production of proinflammatory cytokines and type I IFN in macrophages

To investigate the role of late endosome/lysosome-localized Rab7b in CpG-ODN-induced macrophage activation, we overexpressed or silenced Rab7b in RAW264.7 cells and primary peritoneal macrophages. We found that overexpression of Rab7b in macrophages significantly impaired CpG-ODN-induced mRNA expression of TNF-α, IL-6, IFN-α, and IFN-β in RAW264.7 cells (Fig. 2A) and also in primary peritoneal macrophages (Fig. 2B). We also investigated whether Rab7b silencing could promote TLR9-triggered production of proinflammatory cytokine and IFN-β in macrophages. As shown in Fig. 3A, endogenous Rab7b mRNA expression was significantly increased in Rab7b−/− bone marrow-derived macrophages when they were stimulated with CpG-ODN, while the CpG-ODN-induced expression of TNF-α and IL-6 in Rab7b−/− macrophages was significantly lower than that in Rab7b+/+ macrophages (Fig. 3B). These results suggest that Rab7b plays a critical role in TLR9 signaling and in the production of proinflammatory cytokines and type I IFN.

The online version of this article contains supplemental material.

FIGURE 3. Rab7b knockdown increases production of proinflammatory cytokines and IFN-β in macrophages stimulated with CpG-ODN. A, Mouse primary peritoneal macrophages were transfected with Rab7b siRNA or control small RNA (con siRNA). Forty-eight hours later, the efficiency of silencing was examined by quantitative PCR. B, RAW264.7 cells were transfected with Rab7b siRNA or control small RNA. Thirty-six hours later, the cells were transfected with siRNA-targeted site mutant Rab7b or mock vector. Thirty-six hours later, the cells were stimulated with 0.3 μM CpG-ODN for 8 h, and then CpG-ODN-induced TNF-α and IFN-β in the supernatants were measured by ELISA. C, RAW264.7 cells were transfected with Rab7b siRNA or control small RNA. Thirty-six hours later, the cells were stimulated with CpG-ODN for 8 h, and then CpG-ODN-induced TNF-α and IFN-β in the supernatants were measured by ELISA. Similar results were obtained in three independent experiments. Data are shown as mean ± SD of three independent experiments. *, p < 0.05; **, p < 0.01.
expression in primary macrophages was significantly suppressed to 25% by siRNA. Consistently, Rab7b silencing significantly increased CpG-ODN-induced TNF-α, IL-6, and IFN-β production in primary peritoneal macrophages (Fig. 3B). To exclude the possible off-target effects of RNA silencing, we also constructed specific mutant forms of Rab7b that contained five synonymous mutations on siRNA target position of the mRNA to prevent the association of messenger RNA and siRNA. We found that transfecting these mutants to the Rab7b stably silenced RAW264.7 cells or Rab7b-siRNA directly leads to ubiquitination and degradation, and then NF-κB is activated and translocates into the nucleus as a transcription factor. Thus, we assessed CpG-ODN-induced NF-κB luciferase activity in HEK293 cells cotransfected with or without 50 ng of TLR9-expressing plasmid, 50 ng of pGL3.5 × xB-luciferase reporter plasmid, 10 ng of pTK-Renilla-luciferase, and 90 ng of Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture and indicated time of CpG-ODN stimulation, luciferase activity was measured and normalized by Renilla luciferase activity. As shown in Fig. 4B, and phosphorylation of IκBα (Fig. 4B). Interestingly, NF-κB luciferase reporter assay showed that Rab7b overexpression could not inhibit MyD88-mediated NF-κB activity in HEK293 cells (Fig. 4C). Then we cotransfected TLR9 and Rab7b into HEK293 cells and detected NF-κB activity by luciferase reporter gene system. As shown in Fig. 4D, overexpression of Rab7b could significantly attenuate NF-κB activity during CpG-ODN challenge. Similarly, the negative regulatory effect of Rab7b on IFN regulatory factor (IRF) 7 activation was also found in HEK293 cells cotransfected with Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture and indicated time of CpG-ODN stimulation, luciferase activity was measured and normalized by Renilla luciferase activity. Similar results were obtained in three independent experiments. Data are shown as mean ± SD of three independent experiments. *p < 0.05; **p < 0.01.

**FIGURE 4.** Ovreexpression of Rab7b inhibits TLR9 signaling pathway in macrophages. HA-Rab7b stably overexpressed RAW264.7 cells and control cells were treated with 0.3 μM CpG-ODN for the indicated time. Phospho-ERK, JNK, and p38 (A), as well as phospho-IκBα and total IκBα (B) were detected by immunoblot. Similar results were obtained in three independent experiments. C, HEK293 cells were cotransfected with or without 50 ng of MyD88-expressing plasmid, 50 ng of pGL3.5 × xB-luciferase reporter plasmid, 10 ng of pTK-Renilla-luciferase, together with indicated amount of Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture, luciferase activity was measured and normalized by Renilla luciferase activity. D, HEK293 cells were cotransfected with or without 50 ng of TLR9-expressing plasmid, 50 ng of pGL3.5 × xB-luciferase reporter plasmid, 10 ng of pTK-Renilla-luciferase, and 140 ng of Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture and indicated time of CpG-ODN stimulation, luciferase activity was measured and normalized by Renilla luciferase activity. E, Luciferase assay of IRF7 activation in HEK293 cells cotransfected with or without 50 ng of TLR9-expressing plasmid, 50 ng of plasmid-expressing Gal4-IRF7, 50 ng of Gal4 reporter plasmid, 10 ng of pTK-Renilla-luciferase, and 90 ng of Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture and indicated time of CpG-ODN stimulation, luciferase activity was measured and normalized by Renilla luciferase activity. Similar results were obtained in three independent experiments. Data are shown as mean ± SD of three independent experiments. *p < 0.05; **p < 0.01.

**Rab7b suppresses TLR9 signaling pathway in macrophages**

It is well known that activation of both MAPK and NF-κB pathways is necessary for CpG-ODN-induced cytokine production (3). First, we analyzed the phosphorylation of ERK1/2, JNK1/2, and p38 in Rab7b-overexpressing macrophages and found that Rab7b overexpression could inhibit TLR9-initiated activation of ERK1/2, JNK1/2, and p38 (Fig. 4A). Meanwhile, silencing of Rab7b slightly promoted activation of these pathways in macrophages stimulated with CpG-ODN (data not shown).

IκBα is the inhibitor of NF-κB, and phosphorylation of IκBα directly leads to ubiquitination and degradation, and then NF-κB is activated and translocates into the nucleus as a transcription factor. Thus, we assessed CpG-ODN-induced IκBα phosphorylation and NF-κB activity in Rab7b-overexpressing RAW264.7 cells. We found that Rab7b overexpression remarkably decreased the phosphorylation level of IκBα (Fig. 4B). Interestingly, NF-κB luciferase reporter assay showed that Rab7b overexpression could not inhibit MyD88-mediated NF-κB activity in HEK293 cells (Fig. 4C). Then we cotransfected TLR9 and Rab7b into HEK293 cells and detected NF-κB activity by luciferase reporter gene system. As shown in Fig. 4D, overexpression of Rab7b could significantly attenuate NF-κB activity during CpG-ODN challenge. Similarly, the negative regulatory effect of Rab7b on IFN regulatory factor (IRF) 7 activation was also found in HEK293 cells cotransfected with Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture and indicated time of CpG-ODN stimulation, luciferase activity was measured and normalized by Renilla luciferase activity. Similar results were obtained in three independent experiments. Data are shown as mean ± SD of three independent experiments. *p < 0.05; **p < 0.01.

**FIGURE 4.** Ovreexpression of Rab7b inhibits TLR9 signaling pathway in macrophages. HA-Rab7b stably overexpressed RAW264.7 cells and control cells were treated with 0.3 μM CpG-ODN for the indicated time. Phospho-ERK, JNK, and p38 (A), as well as phospho-IκBα and total IκBα (B) were detected by immunoblot. Similar results were obtained in three independent experiments. C, HEK293 cells were cotransfected with or without 50 ng of MyD88-expressing plasmid, 50 ng of pGL3.5 × xB-luciferase reporter plasmid, 10 ng of pTK-Renilla-luciferase, together with indicated amount of Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture, luciferase activity was measured and normalized by Renilla luciferase activity. D, HEK293 cells were cotransfected with or without 50 ng of TLR9-expressing plasmid, 50 ng of pGL3.5 × xB-luciferase reporter plasmid, 10 ng of pTK-Renilla-luciferase, and 140 ng of Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture and indicated time of CpG-ODN stimulation, luciferase activity was measured and normalized by Renilla luciferase activity. E, Luciferase assay of IRF7 activation in HEK293 cells cotransfected with or without 50 ng of TLR9-expressing plasmid, 50 ng of plasmid-expressing Gal4-IRF7, 50 ng of Gal4 reporter plasmid, 10 ng of pTK-Renilla-luciferase, and 90 ng of Rab7b-expressing plasmid. Total amounts of plasmid DNA were equalized using empty control vector. After 24 h of culture and indicated time of CpG-ODN stimulation, luciferase activity was measured and normalized by Renilla luciferase activity. Similar results were obtained in three independent experiments. Data are shown as mean ± SD of three independent experiments. *p < 0.05; **p < 0.01.
with TLR9 (Fig. 4E). Thus, Rab7b could impair TLR9-initiated signal pathway, and this impairment might function upstream, but not downstream of MyD88.

**Rab7b colocalizes with TLR9 in late endosome/lysosome after TLR9 activation**

Considering that the impairment of TLR9 signaling by Rab7b occurred at upstream, but not downstream of MyD88, we went further to observe whether Rab7b could interact with intracellular TLR9. We first examined the possibility of Rab7b interacting with any molecules in the TLR9 signaling pathway or TLR9 itself. However, we failed to identify any molecule in the TLR9 signaling pathway or TLR9 itself that can directly bind to Rab7b (data not shown).

Then we observed the spatial localizations of Rab7b and TLR9. Rab family proteins target to different subcellular organelles, and the known functions for Rab family proteins are the regulation of myriad-trafficking processes. As shown in Fig. 5A, we used anti-HA mAb and Alexa Fluor 488-conjugated secondary Ab to mark Rab7b and late endosome/lysosome marker LAMP1 and Alexa Fluor 594-conjugated secondary Ab to represent late endosome/lysosome. Rab7b was exactly localized in the LAMP1-positive compartment in RAW264.7 cells. TLR9 interacts and preassociates with high mobility group box 1 in the endoplasmic reticulum in the resting cells before binding to CpG-ODN in macrophages (26). Then we analyzed the TLR9 translocation in RAW264.7 cells stably transfected with HA-tagged Rab7b after 0.3 μM CpG-ODN treatment. TLR9 did not colocalize with late endosome/lysosome marker LAMP1 in resting cells. Thirty minutes after CpG-ODN stimulation, TLR9 was trafficked to late endosome/lysosome and colocalized with LAMP1 (Fig. 5B). Moreover, TLR9 also colocalized with Rab7b in macrophages ~30 min after CpG-ODN stimulation (Fig. 5C). These data suggested a direct linkage between Rab7b localization and TLR9 transport, proposing the possibility that Rab7b may affect the expression and function of TLR9.

**Rab7b promotes the lysosomal degradation of TLR9 in macrophages**

We went further to investigate the mechanisms by which Rab7b could negatively regulate TLR9 signaling in macrophages. It has been reported that TLR9 would undergo the translocation process from endoplasmic reticulum (ER) to a tubular lysosomal compartment after being treated with CpG-ODN (22). It is well known that cellular protein would be finally transported either to endosome for ubiquitination or to lysosome for degradation. TLR9 is an intracellular pattern recognition receptor, and first locates in the ER at rest and then translocates to endosomes or lysosomes after its activation. Rab7b belongs to the small guanosine triphosphatase (GTPase) Ras superfamily, and is predicted to regulate the membrane transport of proteins (20). We have revealed that Rab7b could negatively regulate TLR4 signaling by inhibiting the expression of TLR4 via promotion of TLR4 degradation (21). Therefore, we wondered whether Rab7b could also down-regulate TLR9 expression. We analyzed the expression of total TLR9 protein in

**FIGURE 5.** TLR9 transports to and colocalizes with Rab7b in late endosome/lysosome of macrophages after TLR9 activation. A, RAW264.7 cells were transiently transfected with HA-tagged Rab7b. Forty-eight hours later, cells were immunostained with anti-HA and late endosome/lysosome marker LAMP1 Abs, as well as different secondary Abs. The slides were examined under confocal microscopy, and representative images were presented. B, RAW264.7 cells were treated with or without 0.3 μM CpG-ODN for 30 min. Then cells were immunostained with anti-TLR9 and anti-LAMP1 Abs, as well as different secondary Abs. The representative images were acquired by confocal microscopy. C, RAW264.7 cells were transiently transfected with HA-tagged Rab7b. Forty-eight hours later, the cells were stimulated with 0.3 μM CpG-ODN for indicated period. Then the cells were immunostained with anti-TLR9 and anti-HA Abs, followed by Alexa Fluor 488- and Alexa Fluor 594-labeled secondary Abs, respectively. Representative images were shown (bar = 100 μm).

**FIGURE 6.** Rab7b promotes the degradation of TLR9 in macrophages. A, HA-Rab7b stably overexpressed and mock control cells were stimulated with 0.3 μM CpG-ODN for 1, 2, 4, 8, and 20 h, respectively. TLR9 and HA-Rab7b were detected by immunoblot. B, HA-Rab7b stably overexpressing and mock control cells were pretreated with 10 μM MG132 2 h before and then stimulated with 0.3 μM CpG-ODN for 1, 2, 4, or 8 h, respectively. TLR9 and HA-Rab7b were detected by immunoblot. Data are representative of three separate experiments.
macrophages with Rab7b stable overexpression after being treated with 0.3 μM CpG-ODN for 24 h. We found that Rab7b overexpression significantly inhibited TLR9 expression in macrophages once TLR9 was activated (Fig. 6A). Even the macrophages were resting, i.e., the Rab7b-overexpressing macrophages were not stimulated with 0.3 μM CpG-ODN and the expression of TLR9 protein remained at a relative low level as compared with the mock control. To exclude the possibility that Rab7b mediated TLR9 degradation via an ubiquitin-dependent process, we pretreated Rab7b-overexpressing cells and control cell with 10 μM MG132, the specific inhibitor of proteasome, for 2 h, and then stimulated the cells with CpG-ODN. Western blot assay showed that inhibitor of proteasome could not reverse the Rab7b-mediated degradation of TLR9 (Fig. 6B). Thus, overexpression of Rab7b might promote the lysosomal degradation of TLR9 in macrophages once TLR9 is activated.

Discussion
As the first barrier of host against microbe infection, the innate immune system plays an important role in protecting hosts from invading microbial pathogens and evoking subsequent adaptive immunity (27). Recently, studies have demonstrated that the innate immune pathway was also implicated in the pathogenesis of certain systemic autoimmune diseases, such as rheumatoid arthritis and SLE (28, 29). During the pathogenic process of SLE, TLR9 detects host DNA-Ig complex, and then robust IFN response is triggered (5, 27). Recent studies have shown that DNA-containing immune complexes within SLE serum stimulate pDCs to produce IFN-α, which is mediated cooperatively by TLR9 and CD32 (30). Ligation of TLR9 not only induces abnormal IFN response, but also activates autoreactive B cells to produce specific autoantibody (31–33). Thus, understanding of down-regulation of TLR9-triggered immune response is helpful to design new potential therapeutic approaches to autoimmune diseases. At present, TLR inhibitors, especially those to TLR7 and TLR9, have been considered as potential therapeutic drug for SLE (34). In this study, we demonstrated that Rab7b, as an endogenous TLR9 inhibitor, could suppress the CpG-ODN-initiated ERK, JNK, p38, and NF-κB activities and down-regulate the TLR9-triggered proinflammatory cytokine and type I IFN production. However, Rab7b expression is also suppressed during TLR9 activation; such reduction of the TLR9 suppressor might be the mechanism used by the hosts to avoid suppression of the innate immune response and thoroughly remove invading pathogenic microbes. As the potential therapeutic target for SLE, we should fully understand which signaling is mainly responsible for the regulation of Rab7b and how to promote Rab7b expression so as to suppress TLR9 activation in the future.

Small GTPases of the Rab family were considered to control vesicular formation, movement, and timing of vesicle fusion (17). However, new functions of these Rab family members have been reported recently, especially the potential functions in immune system. It seemed that Rab7 was involved in the growth factor signaling regulating cell nutrient and apoptosis (35). Recently, Rab27a was implicated in Ag cross-presentation and was rapidly recruited to phagosomes only when Ig was present that potentiated the efficiency of Ag cross-presentation to CD8+ T cells. Rab7b is specifically expressed in mononcytic cells and monocyte-derived cells (36). Moreover, our previous findings also gave rise to a view on the down-regulation function of Rab7b in LPS-initiated TLR4 signaling pathway in macrophages (21). Many negative regulators of TLR signaling have been identified these years (10, 37). In this study, we showed evidence to support such a conclusion that Rab7b also negatively regulates CpG-ODN-initiated TLR9 signal transduction triggered from intracellular compartment. Our results showed that overexpression of Rab7b could suppress both MAPK and NF-κB activation, and the production of TNF-α, IL-6, and IFN-β in macrophages stimulated with CpG-ODN. In contrast, production of TNF-α, IL-6, and IFN-β increased in Rab7b-silenced macrophages after CpG-ODN treatment. Our study has identified Rab7b as a potential negative regulator of TLR9 signaling, which might serve as one of the mechanisms for host to protect from overactivation of immune response in inflammation after bacterial infection. Furthermore, we demonstrated that Rab7b overexpression might accelerate the degradation of TLR9 in the lysosomes through colocalization of TLR9 and Rab7b in a late endosome/lysosome-associated intracellular compartment.

There is evidence indicating that TLR9 is localized to the ER of macrophages, and binds directly to CpG DNA in ER. Then CpG DNA is subsequently transported to a lysosomal compartment. Simultaneously, TLR9 redistributes from the ER to CpG DNA-containing structures (22, 26). Lysosome is a kind of acid intracellular compartment in which lots of proteins are finally transported to lysosome for degradation. So, we wondered about the degradation situation of the lysosome-localized TLR9 itself. Rab7b is a late endosome/lysosome-localized GTPase protein and might potentially regulate protein degradations related with late endosome/lysosome. According to our results, Rab7b and TLR9 were both colocalized with the late endosome/lysosome-specific marker LAMP1 30 min after CpG-ODN stimulation, respectively, suggesting that TLR9 was indeed redistributed to late endosome/lysosome with Rab7b, and Rab7b might be involved in this process. We show that Rab7b promotes the lysosomal degradation of TLR9, further indicating that protein degradation is one of the mechanisms used by the innate immune cell to control the signal transduction for avoiding inappropriate activation of TLRs. Through facilitating distribution of receptors to late endosome/lysosome for degradation, Rab7b may participate in other immune reactions, which needs to be investigated in the future.

In conclusion, we demonstrated that Rab7b is a novel negative regulator of TLR9 signaling and can suppress TLR9-triggered TNF-α, IL-6, and IFN-β production in macrophages by promoting TLR9 lysosomal degradation.

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Disclosures
The authors have no financial conflict of interest.

References
Supplemental Materials For

Late endosome/lysosome-localized Rab7b suppresses TLR9-initiated proinflammatory cytokine and type I interferon production in macrophages

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Supplemental Figure 1.

(A) RAW264.7 cells were treated with 0.3μM CpG-ODN for 2, 4, 8, 12 or 24 hours respectively. mRNA expression level of Rab7b was assayed by quantitative PCR. (B) Primary peritoneal macrophages from wild type and TLR9 deficient mice were treated with 0.3μM CpG-ODN for 2, 4, 8, 12 or 24 hours respectively. mRNA expression level of Rab7b was assayed by quantitative PCR. The results were presented as fold expression of Rab7b mRNA to that of β-actin. Data are shown as mean ± SD of three independent experiments.