Synaptotagmin XI Regulates Phagocytosis and Cytokine Secretion in Macrophages

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Synaptotagmin XI Regulates Phagocytosis and Cytokine Secretion in Macrophages

Guillermo Arango Duque,*† Mitsunori Fukuda,‡ and Albert Descoteaux*†

Synaptotagmins (Syts) are a group of type I membrane proteins that regulate vesicle docking and fusion in processes such as exocytosis and phagocytosis. All Syts possess a single transmembrane domain, and two conserved tandem Ca\(^{2+}\)-binding C2 domains. However, Syts IV and XI possess a conserved serine in their C2A domain that precludes these Syts from binding Ca\(^{2+}\) and phospholipids, and from mediating vesicle fusion. Given the importance of vesicular trafficking in macrophages, we investigated the role of Syt XI in cytokine secretion and phagocytosis. We demonstrated that Syt XI is expressed in murine macrophages, localized in recycling endosomes, lysosomes, and recruited to phagosomes. Syt XI had a direct effect on phagocytosis and on the secretion of TNF and IL-6. Whereas small interfering RNA–mediated knockdown of Syt XI potentiated secretion of these cytokines and particle uptake, overexpression of an Syt XI construct suppressed these processes. In addition, Syt XI knockdown secreted TNF and IL-6. Whereas small interfering RNA–mediated knockdown of Syt XI potentiated secretion of these cytokines (e.g., TNF and IL-6) that are responsible for unleash- ing an effective immune response, and for mediating the transition between innate and adaptive immunity (1, 4, 5). In macrophages, the formation and maturation of phagosomes are sequential pro- cesses that necessitate extensive remodeling of the cytoskeleton and exchanges with multiple organelles in a process termed kiss and run (6). The maturation of phagosomes is controlled by Rab proteins, which are a subtype of GTPases, with Rab5 and Rab7 governing fusion events of phagosomes with early and late endosomes, respectively (7). As phagosomes mature, they become highly acidic and acquire a panoply of highly microbicidal mol- ecules, such as cathepsins and other acid hydrolases (8). Late phagosomes acquire markers such as the lysosomal-associated membrane proteins (LAMP)–1 and –2, which are required for acquisition of Rab7 (9) and of microbicidal properties (10). Crucial to the killing of many intracellular pathogens is the genera- tion of reactive oxygen species (ROS) in the phagosome, which is produced mostly by the NADPH oxidase 2 complex (NOX2) (11, 12) and mitochondria (13). The complex, which is normally inactive in the cell, is constituted by six subunits. These subunits are the Ru guanosine triphosphate (GTPase), usually Rac1 or Rac2, and five phagocytic oxidase (phox) subunits. Phox subunits are the membrane-anchored gp91\(^{phox}\) and p22\(^{phox}\), which together form the vesicular flavocytochrome b\(_{556}\) and the cytoplasmic compo- nents p40\(^{phox}\), which interacts with heterodimers of p47\(^{phox}\) and p67\(^{phox}\) (14). The oxidase becomes functional when an activated p40\(^{phox}\)-p47\(^{phox}\)-p67\(^{phox}\) heterotrimer is recruited to a membrane- bound flavocytochrome b\(_{556}\), which then produces ROS by oxi- dizing NADPH to NADP\(^+\) in the cytoplasmic region of gp91\(^{phox}\). This reaction liberates one H\(^+\) ion and two electrons that travel through the flavocytochrome b\(_{556}\) into the phagosomal lumen to react with oxygen molecules and produce superoxide ions. The preponderance of ROS, acid hydrolases, and other microbicidal molecules create a hostile intraphagosomal milieu that fettters the survival of most pathogens. A highly organized orchestration of vesicular trafficking is es- sential for the proper spatiotemporal execution of cytokine se- cretion and phagocytosis. This trafficking is regulated by members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) such as the vesicle-associated membrane protein (VAMP)3, which promotes trafficking of TNF-containing vesicles to the cell membrane and to nascent phagosomes (5, 15). Constitutive exocytosis is responsible for the secretion of TNF and IL-6. SNARE proteins Syntaxin-6, -7, and Vti1b control trafficking from the Golgi complex to recycling endosomes. From recy- cling endosomes, VAMP3-bearing vesicles are delivered to the plasma or phagosome membrane where Syntaxin-4—a cognate SNARE of VAMP3—is present. Although TNF is delivered to forming phagosomes, IL-6 is not (16). Failure to regulate cytokine secretion results in pathologies in which excess TNF and IL-6 leads to chronic inflammation (17, 18).
The action of SNARE is regulated by synaptotagmins (Systs), which are a large group of membrane proteins that regulate vesicle docking and fusion in processes such as the exocytosis of synaptic vesicles and hormones (19), phagocytosis (18, 20), mast cell degranulation, and acrosome exocytosis in sperm cells (21). All Systs possess a single transmembrane domain, and two conserved tandem C2 domains, which were originally identified in protein kinase C (22). Eight members of the Syst family function as Ca2+ sensors of vesicle fusion by virtue of the C2 domains present in these Systs. Systs IV and XI, however, belong to a different Syst family in which a conserved serine in the C2A domain precludes these Systs from binding Ca2+ and phospholipids (19, 23). In fact, murine Systs IV and XI inhibit vesicle fusion (24) and may mediate vesicle trafficking processes that do not depend on Ca2+ (23, 24).

Indeed, Syst IV has been found to regulate synaptic function by negatively regulating brain-derived neurotrophic factor release (25), among others. As of today, a function had not been assigned to Syst XI. Nonetheless, Syst XI exists in different vesicular compartments of the rat pheochromocytoma cell line PC12 (26, 27). It was also discovered that Syst XI binds to KIF1A, a kinesin-related molecular motor in the brain (28), and with the disseveled (Dvl) protein, which plays a major role in the Wnt signaling axis (29). Interestingly, Syst XI is degraded by Parkin, a protein that is often mutated in Parkinson disease. Defective Parkin leads to accumulation of Syst XI at the core of Lewy bodies (30). All these results have led authors to postulate that Syst XI might have a role in modulating synaptic transmission in the brain. In macrophages, only Syst V and Syst VII have been found to be expressed. Syst V is a positive regulator of phagocytosis that regulates acquisition of the V-ATPase to maturing phagosomes (32). We hypothesized that Syst IV and Syst XI, however, belong to a different Syt family in which a conserved serine in the C2A domain precludes these Syts from binding Ca2+ and phospholipids (19, 23). In fact, murine Systs IV and XI inhibit vesicle fusion (24) and may mediate vesicle trafficking processes that do not depend on Ca2+ (23, 24).

Syst XI might have a role in modulating synaptic transmission in the brain. In macrophages, only Syst V and Syst VII have been found to be expressed. Syst V is a positive regulator of phagocytosis that regulates acquisition of the V-ATPase to maturing phagosomes (20, 31), and Syst VII mediates lysosomal membrane delivery to nascent phagosomes (32). We hypothesized that Syst IV and Syst XI may be expressed in macrophages and have an inhibiting function in cytokine secretion and phagocytosis. Our initial experiments revealed that only Syst XI was expressed in macrophages. In this work, we examined the role of Syst XI in macrophages. We showed that this phagosome-associated Syst negatively controls particle ingestion and the release of TNF and IL-6. Although the lack of Syst XI led to augmented particle intake, phagosomes appeared weakened by their impaired recruitment of gp91phox and LAMP-1. We demonstrated that Syst XI knockdown leads to diminished bactericidal activity, likely as a consequence of less microbialide phagosomes.

Materials and Methods

Ethics statement

Mice were manipulated in strict accordance to protocol 0811-09, approved by the Comité Institutional de Protection des Animaux of the INRS-Institut Armand-Frappier. This protocol respects guidelines on good animal practice provided by the Canadian Council on animal care.

Abs and plasmids

The rabbit polyclonal Ab targeting the C2A domain of Syst XI was produced and purified by affinity chromatography (33). The rat monoclonal anti-transferin receptor 1 (TfR1) was obtained from Cedarlane Laboratories. The rat monoclonal anti-early endosomal Ag 1 Ab and the mouse monoclonal anti-gp91phox Ab were from BD Transduction Laboratories. The rat monoclonal anti-lysosome-associated membrane protein 1 (LAMP-1) Ab was developed by J.T. August (ID4B) and purchased through the Developmental Studies Hybridoma Bank at the University of Iowa, and the National Institute of Child Health and Human Development. The rabbit polyclonal Ab against p38 was obtained from Cell Signaling Technology. The FLAG-Syt XI-GFP construct (34) was inserted into the Norl site of the pCIN4 expression vector (35) using conventional cloning techniques.

Cell culture

The mouse macrophage cell line RAW 264.7 and the PC12 neuroendocrine cell line were cultured in complete DMEM (Life Technologies) with t-glutamine, supplemented with 10% heat-inactivated FBS (PAA Laboratories), 10 mM HEPES at pH 7.4, and antibiotics (Life Technologies) in a 37°C incubator with 5% CO2. Bone marrow–derived macrophages (BMMs) were extracted from the bone marrow of 6–8-wk-old female BALB/c mice (Charles River), and differentiated with complete DMEM with L929 cell-conditioned medium (15% v/v) as a source of CSF 1 (36). Prior to the experiments, BMMs were transferred to tissue-culture treated Petri dishes containing complete DMEM with no L929-conditioned medium for 16 h.

Transfections

For small interfering RNA (siRNA) transfections, RAW 264.7 macrophages in the second passage were reverse-transfected in 24-well plates with the lipidfectin RNAMAX Reagent (Life Technologies) according to the manufacturer’s protocol. The final concentration of siRNA was 25 nM in a final volume of 600 μl; incubation in transfection medium lasted 60 h. Prior to the experiments, macrophages were cultured for an extra 6 h in DMEM containing 10% FBS. Macrophages were mock-transfected, transfected with siRNA to GFP (37), or transfected with the ON-TARGETplus SMARTpool siRNA to Syst XI (Thermo Scientific), which contains four Systs with the following target sequences: sequence 1, CGAUCGAC-ACUAAGAAG; sequence 2, GAGAGAGUGUCGCCAGAGU; sequence 3, AUGUCAUGUGAAGCUCUA; sequence 4, GCACAGCUCCGGCA-GUAC. BLAST searches were performed to ensure that these sequences were targeted only the Syst XI mRNA. The pCIN4 plasmid containing the FLAG-Syt XI-GFP construct, or empty pCIN4, was electroporated into RAW 264.7 macrophages (38). Stably transfected macrophages were selected in complete DMEM containing 500 μg/ml G418, and individual clones were expanded and assayed for FLAG-Syt XI-GFP expression by Western blot and confocal immunofluorescence microscopy.

RT-PCR

TRizol (Life Technologies) was used to extract RNA from adherent BMM, RAW 264.7 macrophages, or PC12 cells, per the manufacturer’s instructions. RNA was reverse-transcribed (39), and PCR was performed with a DNA thermal cycler (Perkin-Elmer Corporation, version 2.3) with the following primer pairs, and an annealing temperature of 55°C. Hypoxanthine phosphoribosyltransferase (HPRT): F-AD55: 5’-GGTGGAACAGGGCGCA-GACCTTTGTGG-3’; R-AD56: 5’-GATTCAACTGGGCTCATTTAGGC-3’. Syst IV: F-AD476: 5’-CACCTACCGAAATCTGATGTCGTC-3’; R-AD477: 5’-GACCGACCGCTCAACTACCT-3’; Syst XI: F-AD454: 5’-CAATGCGTGGTTTCGCCAGTAGA-3’; R-AD446: 5’-CTGCAACCGGACCATC-TTGCAG-3’. Samples were run in 1.5% (w/v) agarose gels and the α Imager 3400 (α Innotech Corporation) machine was used to photograph gels.

Cytokine secretion measurements

RAW 264.7 macrophages were stimulated with LPS from Escherichia coli O127:B8 (Sigma) at a final concentration of 100 ng/ml. After stimulation, cell culture supernatants were collected and centrifuged to remove debris. ELISA kits were used as per the manufacturers’ protocols to quantify murine TNF (Ready-SET-Go! Mouse TNFα Kit, eBiosciences) and IL-6 (BD OptEIA, Becton-Dickinson Biosciences) secretion.

Phagosome isolation and phagocytosis assays

For the isolation of purified phagosomes, 40 × 107 RAW 264.7 macrophages were seeded overnight in 150 × 20-mm tissue culture dishes. Cells were then incubated at 4°C for 10 min in the presence of 10 ml complete DMEM containing 200 μl magnetic beads (Stapor) with a diameter of 3 μm. Cells were then transferred to 37°C to trigger internalization for the required phagocytosis time. Cells were then washed three times with cold PBS prior to scraping and collection in cold PBS. Cells were then centrifuged at 2000 rpm for 5 min at 4°C, and the pellet was resuspended in purification buffer (50 ml PBS 10X, 2 ml EDTA at 0.5 M and pH 8, and 445 ml H2O; solution was adjusted to pH 7.2). Cells were centrifuged again at 2000 rpm for 5 min at 4°C. Macrophages were then homogenized in 1 ml of purification buffer with protease inhibitors; cells were lysed with a 1-ml syringe using a 22-gauge needle until 90% were lysed without macrophage breakage (as monitored by light microscopy). Homogenates were centrifuged at 2000 rpm for 5 min at 4°C, and the pellet was resuspended in 1 ml of purification buffer with protease inhibitors. Homogenates were then placed on a magnet for 10 min to isolate phag-
osomes. Thereafter, supernatants were aspirated, and isolated phagosomes were washed with purification buffer containing protease inhibitors. Subsequently, isolated phagosomes were lysed with ice-cold lysis buffer containing 1% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM HCl, and protease and phosphatase inhibitors (Roche). Protein concentrations were measured using the Pierce BCA protein assay (Fisher Scientific).

Zymosan was opsonized with mouse serum (40). For synchronized phagocytosis using opsonized zymosan, macrophages were incubated at 4°C for 15 min using the zymosan-to-cell ratio required by the experiment. Macrophages were washed with cold complete DMEM to remove excess particles, and internalization was triggered by transferring cells to 37°C for the required times (20). Cells were then washed with PBS and stained using the HEMA 3 stain set (Fisher) or prepared for confocal immunofluorescence microscopy.

Bacteria-killing assays

RAW 264.7 macrophages transfected with siRNA were infected with nonopsonized E. coli DH1 (OD<sub>600</sub> = 0.6) at a ratio of 20:1. Infections were carried out according to the protocol outlined by Hamrick et al. (41). Bacteria were added in 20 µl PBS, and plates were centrifuged at 1000 × g for 1 min. Next, plates were incubated at 37°C for 20 min prior to four washes with 1 ml PBS. Complete DMEM with 5 µg/ml of gentamicin (Life Technologies) was then added for 20 min (zero time point) or for an additional 4 h. After these time points, macrophages were washed once with 0.5 ml of PBS and lysed with a solution of 1% Triton X-100 (v/v) in PBS. Lysates were diluted, plated in agar plates, and incubated for 18 h at 37°C. Bactericidal activity was assessed by counting colonies in agar plates, and results were expressed as the log<sub>10</sub> of CFUs per milliliter. Infections were completed in 24-well plates in triplicate.

Confocal immunofluorescence microscopy

Macrophages in coverslips were fixed with 2% paraformaldehyde (Canemco and Miravac) for 10 min. Thereafter, cells were permeabilized and blocked for 15 min with a solution composed of 0.1% Triton X-100, 1% BSA, 2% goat serum, 6% nonfat milk, and 50% FBS. This was followed by a 2-h incubation with primary Abs diluted in PBS. Next, cells were incubated with the appropriate combination of secondary Abs (anti-rabbit AlexaFluor 488, anti-rat 568, and anti-mouse 568; Molecular Probes), cholera toxin subunit B linked to AlexaFluor 594 (Ctx) to stain lipid rafts (Molecular Probes), and DRAQ5 to stain DNA (Biostatus) for 30 min. Coverslips were washed three times with PBS after every step. After staining, coverslips were mounted on glass slides with Fluoromount-G (Southern Biotechnology Associates) and sealed with nail polish. Macrophages were imaged with the oil immersion 63× objective of an LSM 780 microscope (Carl Zeiss Microimaging). Images were taken in simultaneous scanning mode and processed via the ZEN 2011 software (Carl Zeiss Microimaging).

SDS-PAGE and Western blotting

Prior to lysis, adherent macrophages were washed with PBS containing 1 mM sodium orthovanadate (Sigma). Cells were scraped in the presence of lysis buffer containing protease and phosphatase inhibitors. After a 10-min incubation on ice, lysates were sonicated and centrifuged for 10 min to remove insoluble matter. After protein quantification, 30 µg of sample was

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**FIGURE 1.** Syt XI is expressed in macrophages. (A) Syt XI, but not Syt IV, is expressed in macrophages as demonstrated by RT-PCR. (B) As observed via immunofluorescence microscopy, BMMs express Syt XI in vesicles distributed throughout the cell and accumulate at the extremities of long protrusions emanating from the cytoplasm (inset). (C) RAW 264.7 macrophages expressing a FLAG-Syt XI-GFP construct. Results are representative of at least three independent experiments. Original magnification ×63.

**FIGURE 2.** Syt XI associates with recycling endosomes and lysosomes. In resting BMMs, Syt XI (green) colocalized extensively with Tfr1 and LAMP1 (red), which are markers of early/recycling endosomes and lysosomes, respectively. (A) Resting BMMs were fixed, immunostained with the Abs combinations shown, and stained with Ctx and DRAQ5 to demarcate lipid rafts (red) and nuclei (blue), respectively. (B) RAW 264.7 macrophages stably expressing the FLAG-Syt XI-GFP construct were fixed and stained with the indicated Abs. Cells were visualized by confocal immunofluorescence microscopy. Colocalized pixels (mask) are shown. Results are representative of at least three independent experiments. Original magnification ×63.
boiled in SDS sample buffer, migrated in 10% SDS-PAGE gels, and analyzed by Western blotting (20).

Data analysis

Statistical significance was assessed using an unpaired two-tailed Student t test (*p < 0.05, **p < 0.01, ***p < 0.001). Error bars in the figures represent SEM. Graphs were plotted with Microsoft Excel or SigmaPlot.

Results

Macrophages express Syt XI

To date, Syt IV and Syt XI have been found to be expressed only in neuronal tissues. The capacity of these Syts to inhibit vesicle fusion (24, 42) prompted us to assay the expression of these proteins in macrophages, because vesicle fusion is essential for processes such as cytokine secretion and phagocytosis (1). By using RT-PCR, we assayed the expression of Syt IV and Syt XI in BMM, RAW 264.7 macrophages, and in the neuroendocrine cell line PC12. We found that both types of macrophages expressed Syt XI, but not Syt IV (Fig. 1A). Next, we immunostained macrophages with an Ab-specific for the C2A domain of Syt XI. Immunofluorescence revealed that Syt XI was expressed in vesicles distributed in the cytoplasm of BMM (Fig. 1B); interestingly, Syt XI accumulated at the extremities of long protrusions that BMM sometimes extended (see inset). Expression of a FLAG-Syt XI-GFP construct in RAW 264.7 macrophages displayed a vesicular distribution pattern similar to that of endogenous Syt XI in BMM (Fig. 1C). Based on these data, we pursued further the characterization of Syt XI.

Syt XI localizes to recycling endosomes and lysosomes

Syt XI associates with the Golgi apparatus of nonstimulated PC12 cells (26) and with the perinuclear region and neurite tips of nerve grown factor–treated PC12 cells (27). We hypothesized that Syt XI could have a similar vesicular distribution in macrophages. We used confocal immunofluorescence microscopy to image resting macrophages costained with Syt XI, DNA, and established markers of lipid rafts (Ctx) (43), early/recycling endosomes (Tfr1) (44), early endosomes (early endosome Ag 1) (45), and lysosomes (LAMP-1) (44). In BMMs, we observed that Syt XI colocalized mostly with Tfr1 and LAMP-1 (Fig. 2A), indicating that Syt XI was present in recycling endosomes and lysosomes. A highly similar colocalization pattern was observed for FLAG-Syt XI-GFP in RAW 264.7 macrophages (Fig. 2B). These findings suggested a possible role for Syt XI in the processes of phagocytosis and cytokine secretion, because recycling endosomes traffic cytokines to the cell surface (5, 16) while providing membrane for phagosome formation (5, 16, 45).

FIGURE 3. Syt XI is recruited to early phagosomes. (A) RAW 264.7 macrophages are temporarily enriched in Syt XI, 1 h after phagocytosis. We performed synchronized phagocytosis on RAW 264.7 macrophages with magnetic beads and isolated phagosomes by magnetic force. PC12 lysate was used as a positive control; the lower band in the PC12 lane is nonspecific. Phagosomal fractions were analyzed for Syt XI content via Western blot. (B) In BMMs, recruitment of Syt XI to phagosomes containing opsonized zymosan (10:1 ratio) occurred early and decreased significantly 1 h after internalization, as evidenced by confocal immunofluorescence microscopy. Recruitment was quantified on the rightmost panel of (B). (C) Syt XI colocalized with Tfr1-positive recycling endosomes at the phagosome 15 min and 1 h after internalization. This experiment was repeated twice in triplicate, and quantifications were performed for 100 phagosomes per triplicate. ***p ≤ 0.001. Original magnification ×63.
46); however, lysosomes also provide membrane and antimicrobial effectors to the maturing phagosome (2, 10, 32).

**Phagosomes recruit Syt XI**

The involvement of Syts V and VII in phagocytosis (20, 32), and the finding that Syt XI is found in recycling endosomes and lysosomes, prompted us to investigate the association of Syt XI to phagosomes. To assess phagosomal recruitment of Syt XI, we isolated magnetic bead phagosomes from RAW 264.7 macrophages (47). Purified phagosomes were lysed, and protein extracts were analyzed by Western blot (Fig. 3A). We observed that Syt XI was enriched in phagosomes 1 h after internalization. However, Syt XI levels decreased after 4 h of bead internalization. To elucidate the phagosomal recruitment of Syt XI, we incubated BMM with opsonized zymosan at a ratio of 10 particles to 1 macrophage. Macrophages were incubated at 4°C to synchronize phagocytosis prior to particle internalization at 37°C for the indicated time points. Cells were then fixed, stained for Syt XI and DNA, and imaged with confocal microscopy. We observed that 15 min after internalization, 55.7% (± 1) of phagosomes were positive for Syt XI, whereas 1 h after internalization, 27.6% (± 2.4) of phagosomes were positive (Fig. 3B). In addition, we observed that Syt XI colocalized with recycling endosomes at the phagocytic cup (Fig. 3C). Given the crucial roles of recycling endosomes in phagocytosis and cytokine secretion (5, 15), this observation strengthened our hypothesis about a role for Syt XI in these processes. Together, these data showed that Syt XI is recruited to early phagosomes, and that such recruitment diminishes with time.

**Knockdown of Syt XI leads to an increase in cytokine secretion and phagocytosis**

Given that Syt XI is found in recycling endosomes and that it is recruited to the phagosome, we attempted to assay the role of Syt XI in the secretion of TNF and IL-6 and in phagocytosis. To this end, we knocked down Syt XI by transfecting RAW 264.7 macrophages with a pool of three siRNAs; this resulted in ~70% decrease in Syt XI levels in total cell lysates and in decreased immunostaining (Fig. 4A, 4B). After siRNA transfection, we stimulated macrophages with LPS for various time points and quantified the secretion of TNF and IL-6 via ELISA analysis of culture supernatants. siRNA-mediated knockdown of Syt XI resulted in a notable increase in TNF and IL-6 secretion, especially after 12 h of stimulation (Fig. 4C, 4D). To assay phagocytosis, siRNA-treated macrophages were synchronously fed with opsonized zymosan particles in various proportions. Syt XI knockdown led to an increased percentage of macrophages ingesting at least one particle, regardless of the cell-to-particle ratio used (Fig. 4E). These data demonstrate that decreased Syt XI levels lead to an increase in cytokine secretion from LPS-stimulated macrophages and to augmented phagocytosis.

**Overexpression of Syt XI leads to a decrease in cytokine secretion and phagocytosis**

To confirm the results that we obtained by using siRNA, we overexpressed Syt XI in RAW 264.7 macrophages. We constructed stable cell lines expressing FLAG-Syt XI-GFP in the pcIN4 vector, and expression was assessed by analysis of total cell lysates by Western blot and by immunofluorescence (Figs. 1C, 5A). For subsequent experiments, we used two independent clones (G and H) to eliminate possible clonal effects. LPS stimulation of macrophages overexpressing FLAG-Syt XI-GFP led to a marked decrease in TNF and IL-6 secretion (Fig. 5B, 5C). Moreover, when these macrophages were fed synchronously with opsonized zymosan particles, a decreased phagocytic index was observed (Fig. 5D). Together with the siRNA data, our findings indicate that Syt XI negatively regulates the processes of TNF and IL-6 secretion and phagocytosis.

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**FIGURE 4.** Knockdown of Syt XI leads to increased cytokine secretion and phagocytosis.

Knockdown of Syt XI in RAW 264.7 macrophages was evaluated by Western blot and by confocal immunofluorescence. (A) RAW 264.7 macrophages were subjected to reverse transfection with Lipofectamine RNAiMAX, lysed, and Syt XI levels were analyzed by Western blot. (B) Macrophages were fixed, immunostained for Syt XI, and visualized by confocal immunofluorescence microscopy. White asterisks denote the location of cells in the panels. (C and D) Transfected macrophages were stimulated with LPS for the indicated time points, and cytokine secretion in culture supernatants was assessed by ELISA. Results represent the mean of two independent experiments performed in triplicate. (D) Synchronized phagocytosis with different ratios of opsonized zymosan was performed after transfection. After 10 min of particle internalization, macrophages were washed and stained via the HEMA-3 kit. The percentage of macrophages with at least one ingested particle was quantified for at least 100 macrophages via light microscopy. Results represent the mean of three independent experiments performed in triplicate. Original magnification ×63. *p ≤ 0.05, **p ≤ 0.01.
cytoplasm, as well as in contouring phagosomes of BMM (Fig. 6A, 6B). These observations prompted us to assay the effect of Syt XI knockdown on the recruitment of gp91phox and LAMP-1 to the phagosome. After siRNA treatment of RAW 264.7 macrophages, synchronized phagocytosis with opsonized zymosan was performed and cells were fixed, stained, and visualized by confocal microscopy. After quantification, we found that cells treated with Syt XI siRNA recruited significantly less gp91phox and LAMP-1 to the phagosome (Fig. 6C, 6D). Given that gp91phox and LAMP-1 have profound effects on the microbicidal capacity of the phagosome (10, 12), our findings indicated that Syt XI could have a direct effect on the ability of macrophages to control intracellular pathogens.

**Knockdown of Syt XI leads to increased intracellular survival of E. coli**

Syt XI knockdown led to an increase in phagocytosis and to phagosomes that showed decreased recruitment of gp91phox and LAMP-1. These observations prompted us to hypothesize that macrophages with decreased Syt XI levels formed phagosomes with a weakened capacity to kill intracellular pathogens. To test this hypothesis, mock- or siRNA-treated macrophages were infected with nonopsonized E. coli DH1. After infection, cells were lysed, and diluted lysates were plated in agar plates. After the zero time point, we found that there were significantly more (p < 0.01) CFUs from macrophages treated with siRNA to Syt XI compared with macrophages treated with siRNA to GFP (Fig. 7). This finding is probably due to an initial increase in phagocytosis as observed for zymosan-fed macrophages (Fig. 4E). Remarkably, macrophages treated with siRNA to Syt XI were not able to clear E. coli 4 h after infection (Fig. 7); indeed, a small increase in the number of CFU was observed from the zero to the 4-h time point. We concluded that Syt XI regulates the microbicidal activity of the phagosome.

**Discussion**

In this study, we characterized a member of the Syt family whose function had not been elucidated previously. Using macrophages, we determined that Syt XI is a vesicular protein that negatively modulates the secretion of TNF and IL-6 and phagocytosis. Although particle uptake is increased upon Syt XI knockdown, the phagosomes that are formed recruit defectively markers that are necessary for microbicidal activity in phagosomes. We showed that such phagosomes are less adept at killing bacteria. Collectively, our results identify Syt XI as a novel regulator of macrophage function.

The finding that Syt XI, and not Syt IV, is expressed in macrophages implies that only one Syt of this subfamily is required to exert a negative regulatory function in macrophage vesicular trafficking. In contrast, PC12 cells express both Syt IV and Syt XI. Although Syt IV has been characterized (25), the function of Syt XI in neuronal cells remains unknown. The association of Syt XI to recycling endosomes and lysosomes was an indication that Syt XI is possibly implicated in cytoskeleton secretion and phagocytosis, because recycling endosomes are an important distribution point for the passage of cytokines to the cell surface, and both recycling endosomes and lysosomes contribute membrane to the developing phagosome (5). Recruitment of Syt XI to the phagosome also prompted us to determine the role of Syt XI in phagocytosis. Syt XI was present more abundantly during the early stages of phagocytosis, which implicates Syt XI in early phagocytic events. We used two complementary approaches to determine that Syt XI was a negative regulator of both cytokine secretion and phagocytosis. Knockdown of Syt XI via siRNA showed that decreased Syt XI expression led to augmented TNF

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**FIGURE 5.** Overexpression of Syt XI leads to decreased cytokine secretion and phagocytosis. (A) RAW 264.7 macrophages were stably transfected with the FLAG-Syt XI-GFP construct in the pCIN4 vector. Expression of this construct was assessed via Western blot, using an Ab-recognizing GFP. The bands under the RAW 264.7 and pCIN4 (empty) lanes are nonspecific, and α denotes degradation products. In both FLAG-Syt XI-GFP clones, the expression pattern is similar, as visualized by confocal microscopy (see Fig. 1C). (B and C) Stably transfected macrophages were stimulated with LPS for the indicated durations. ELISA was used to quantify cytokine secretion in culture supernatants. (D) Opsonized zymosan, in different proportions, was fed to stably transfected macrophages. Ten minutes after internalization, particle internalization was quantified for at least 100 macrophages. All results shown are the mean of three independent experiments performed in triplicate.

**Syt XI regulates the recruitment of gp91phox and LAMP-1 to the phagosome**

After having established that Syt XI was a negative regulator of particle uptake in macrophages, we hypothesized that the absence of Syt XI could also affect the recruitment of proteins that affect phagosomal maturation. Two of these proteins, namely gp91phox and LAMP-1, affect the ability of the phagosome to produce ROS and acquire lysosomal characteristics, respectively. Interestingly, Syt XI colocalized with both of the aforementioned proteins in the developing phagosome (5). Recruitment of Syt XI to the phagosome also prompted us to determine the role of Syt XI in phagocytosis. Syt XI was present more abundantly during the early stages of phagocytosis, which implicates Syt XI in early phagocytic events. We used two complementary approaches to determine that Syt XI was a negative regulator of both cytokine secretion and phagocytosis. Knockdown of Syt XI via siRNA showed that decreased Syt XI expression led to augmented TNF
and IL-6 secretion from stimulated macrophages, especially after 12 h of stimulation; phagocytosis was increased as well. The effect of Syt XI on phagocytosis did not depend on particle load, in contrast to Syt V and Syt VII (20, 32). To further substantiate these results, overexpression of a Syt XI construct diminished levels of secretion and phagocytosis. The role of Syt XI in macrophage trafficking resembles the negative role that Syt II has on the Ca2+-dependent exocytosis of lysosomes (48), and on MHC class II presentation in mast cells (49). Although Syt XI and Syt II belong to different Syt subfamilies that have different properties, both of these Syts negatively regulate exocytic processes in cells of the innate immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system.

There are a number of possible mechanisms by which Syt XI could negatively regulate vesicular trafficking. It has been shown that delivery of TNF-containing vesicles requires the fusion of VAMP3-bearing vesicles with cognate SNAREs, such as Syntaxin-4, at the plasma membrane and at the phagocytic cup (15). In light of the fact that Syt XI inhibits Ca2+-triggered vesicle fusion in liposome fusion assays, Syt XI might hinder the formation of SNARE complexes by sterically hindering interactions between Q- and R-SNAREs. In addition, Syt XI could affect the recycling of vesicles once they fuse to the plasma membrane. The NSF-SNAP complex, which aids in vesicle recycling by dissociating SNARE complexes (50), could be regulated by a mechanism by which Syt XI delays the liberation of SNARE complexes and slows down vesicle traffic as a result. In addition, it has been shown that Syt XI acts as a receptor for the molecular motor KIF1A in neurons (28). If KIF1A, or a related motor, is involved in the exocytosis of cytokine-containing vesicles, or in the focal

![Figure 6](http://www.jimmunol.org/)
delivery of membrane to phagosomes, Syt XI could act as a negative regulator of KIF1A-mediated delivery of vesicles. In addition, it is possible for Syt XI to bind to other molecular motors in macrophages. Because Syt XI has not been found to bind SNAREs, a mechanism for Syt XI function on cytokine secretion action could also depend on the regulation of signaling pathways that regulate cytokine production and release. Future research will determine how Syt XI regulates vesicular trafficking associated with phagocytosis and cytokine secretion.

Aside from augmenting phagocytosis, the absence of Syt XI led to the formation of phagosomes that failed to acquire phagosomal features. gp91phox and LAMP-1 are recruited to phagosomes from recycling endosomes and lysosomes, respectively (9, 51). We found that both gp91phox and LAMP-1 colocalize with Syt XI on endosomes, lysosomes, and phagosomes. In the absence of Syt XI, we showed that recruitment of both gp91phox and LAMP-1 to phagosomes was impaired, indicating that Syt XI participates in the control of phagolysosome biogenesis. Consistent with a role for Syt XI in the control of phagosome maturation, we observed that the ability of macrophages to kill E. coli was reduced in the absence of Syt XI. It is possible that Syt XI partners with other vesicular regulators to modulate the delivery of recycling endosomes and lysosomes containing gp91phox and LAMP-1 to the phagosome. Future experiments will address the mechanisms by which Syt XI regulates the delivery of effectors to phagosomes.

Several intracellular microbes live in remodeled vacuoles that promote their survival and propagation (52, 53). Many of these pathogens have evolved strategies to impede phagosome maturation by interfering with the formation of ROS or by preventing acquisition of lysosomal effectors to the phagosome (52–55). Our findings suggest that Syt XI could be a target for pathogens that have the capacity to exclude Syt XI from their phagosome. Parasites of phagocytes could destroy Syt XI to provoke an overproduction of cytokines that would then attract more phagocytes and facilitate parasite dissemination. The absence of Syt XI could also contribute to pathogen survival by impeding recruitment of phagosomal effectors such as gp91phox and LAMP-1 and thus prevent killing of the microorganism.

In summary, our results establish Syt XI as a novel negative regulator of cytokine secretion and phagocytosis. Moreover, Syt XI controls phagolysosome biogenesis, likely by modulating the delivery of antimicrobial effectors to the phagosome. Because of the similarity between Syt XI and Syt IV, it is reasonable to infer that Syt XI exerts analogous functions in the trafficking of other cells, including those of the immune system.

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Disclosures
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References