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Vesicle-Associated Membrane Protein-8/Endobrevin Negatively Regulates Phagocytosis of Bacteria in Dendritic Cells

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Phagocytosis is a specialized mechanism used by mammalian cells, particularly the cells of the immune system, such as dendritic cells (DC) and macrophages, to protect the host against infection. The process involves a complex cascade of pathways, from the ligation of surface receptors of phagocytes with components of the microorganism’s surface, formation of phagosomes and subsequently phagolysosomes, to the eventual presentation of foreign Ags. Vesicle-associated membrane protein (VAMP)-8/endobrevin has been shown previously to function in the endocytic pathways. Our results showed that VAMP-8 colocalized with lysosome-associated membrane protein-2, and a significant amount of VAMP-8 was recruited to the phagosomes during bacterial ingestion. However, overexpression of VAMP-8 significantly inhibited phagocytosis in DC. We also found that the phagocytic activity of VAMP-8−/− DC was significantly higher than wild-type VAMP-8+/+ DC, thus further confirming that VAMP-8 negatively regulates phagocytosis in immature DC. The Journal of Immunology, 2008, 180: 3148–3157.
C57BL/6 mice (c–e) were similarly marrow-derived primary DC from Cy3-conjugated anti-rabbit IgG. Bone FITC-conjugated anti-rat IgG and LAMP-2 (monoclonal), followed by clonal or VAMP-3 (polyclonal) and with Abs against VAMP-8 (polyclonal). VAMP-8 knockout mice were generated as previously described (19).

Generation of VAMP-8 knockout mice

VAMP-8 knockout mice were generated as previously described (19).

Cloning of VAMP-3, VAMP-7, and VAMP-8 gene

 Extraction of plasmid was based on the QIAprep Spin Miniprep kit. Plasmids extracted from Escherichia coli containing mouse VAMP-8-ligated pCMV-SPORT6 vector (Mammalian Gene Collection (MGC) ID: 13842, Integrated Molecular Analysis of Genomes and their Expression (IMAGE) ID: 4205509), human VAMP-3-ligated pDNR-LIB vector (MGC:12463, IMAGE:3681914), and human VAMP-7-ligated pCMV-SPORT6 vector (MGC:64832, IMAGE:6503665) were subjected to PCR using Pfu polymerase. Forward primers were designed with an EcoRI restriction sequence and reverse primer with XbaI sequence. The following VAMP primers were used: VAMP-8, (forward) 5′-GGAATTCATGGAGGAGGCCAG-3′ and (reverse) 5′-GCTCTAGAGCTTAATGGGGATG-3′; VAMP-3, (forward) 5′-GGAATTCATGCTTACAGGTCCAACTGC-3′ and (reverse) 5′-GCTCTAGAGCTTAATGGGGATG-3′; VAMP-7, (forward) 5′-GGAATTCATGGCGATTCTTTTTGCT-3′ and (reverse) 5′-GCTCTAGAGCTTAATGGGGATG-3′; VAMP-8, (forward) 5′-GGAATTCATGCTTACAGGTCCAACTGC-3′ and (reverse) 5′-GCTCTAGAGCTTAATGGGGATG-3′. The desired DNA band was excised and purified using QIAquick Gel Extraction, and subjected to EcoRI and XbaI digestion. The VAMP-3/VAMP-7/VAMP-8 insert was ligated to EcoRI- and XbaI-digested pMyc plasmid with a double Myc tag at the 5′ terminal. Transformation was performed and the clones were selected using 200 µg/ml ampicillin. Sequences were confirmed via sequencing.

Preparation of primary DC

Primary mouse bone marrow-derived DC were prepared according to the method reported previously (20, 21). Briefly, cells were flushed from the long bones and cultured in complete DMEM with 10% FBS containing 20 ng/ml GM-CSF (day 0). Cells were fed with fresh complete DMEM containing 20, 15, and 10 ng/ml GM-CSF on day 2, 4, and 6, respectively. The cells were used on either day 5, 6, or 7.

Materials and Methods

Abs and enzymes

The Abs used were purchased commercially from the following manufacturers: VAMP-3 (Synaptic Systems), VAMP-8 (Synaptic Systems), Syn-16 (Synaptic Systems), lysosome-associated membrane protein (LAMP)-2 (BD Biosciences), monoclonal anti-c-myc (Calbiochem), polyclonal anti-c-myc (Santa Cruz Biotechnology), CD204 (Sertotec), CD36 (BD Biosciences), epidermal growth factor receptor (Upstate Biotechnology), and β-actin (Sigma-Aldrich). Fluorescent-conjugated secondary Abs were from Jackson ImmunoResearch Laboratories. The restriction endonucleases EcoRI and XbaI, T4 DNA ligase and Pfu polymerase were purchased from Promega.

Abs and enzymes

FIGURE 1. Majority of VAMP-8 is localized to the late endosomal compartments in DC. DC2.4 cells (a and b for VAMP-8, f and g for VAMP-3) were fixed and costained with Abs against VAMP-8 (polyclonal) or VAMP-3 (polyclonal) and LAMP-2 (monoclonal), followed by FITC-conjugated anti-rat IgG and Cy3-conjugated anti-rabbit IgG. Bone marrow-derived primary DC from C57BL/6 mice (c–e) were similarly stained for VAMP-8 and LAMP-2. Images were taken using Olympus Fluoview 500 (a and b, f and g) or Zeiss LSM 510 Meta (c–e). a, Z-scaling = 0.39 µm, Z-stack size = 13.6 µm (n = 35 slices); b, Z-scaling = 0.39 µm, Z-stack size = 15.2 µm (n = 39 slices); f and g, Z-scaling = 0.39 µm, Z-stack size = 16.0 µm (n = 41 slices). Stereo three-dimensional rendering and X-Z/Y-Z projection were performed using the volocity visualization software from Improvision. Scale bar represents 10 µm.
Phagocytosis assay

The phagocytosis assay is based on the Vybrant Phagocytosis Assay kit, with three modifications made to the protocol. First, 100 µl of K12 fluorescence E. coli bioparticles was mixed with 100 µl of medium to provide the cells with better conditions for engulfment. Second, the phagocytosis incubation time was reduced from 3 to 2 h to avoid the phagocytic activities of cells to be compared from reaching a plateau phase. Third, quenching time was doubled from 1 to 2 min to ensure extracellular particles were sufficiently quenched. Cells seeded into a 96-well ELISA plate at a concentration of 1–2 × 10^5 cells/well were allowed to adhere for 4 h to the bottom of the well, after which the fluorescence-conjugated E. coli bioparticles were added and phagocytosis was allowed to proceed for 2 h. The bioparticles were removed and the wells were added with trypan blue for 2 min to quench extracellular bioparticles. The trypan blue was then removed and the amount of bioparticles engulfed by the cells was quantitatively measured using either SpectraMax Gemini EM ELISA plate reader at 480 nm excitation and 520 nm emission or Tecan GENios ELISA plate reader at 485 nm excitation and 535 nm emission.

Isolation of phagosomes

DC2.4 cells were allowed to internalize GFP-E. coli (0.75 × 10^9 particles/ml) for 1 h. The cells were then washed three times with ice-cold PBS to remove extracellular bacteria and trypsinized for 10 min at room temperature. Cells were centrifuged at 100 × g (720 rpm on Beckman Coulter Allegra X-22R Centrifuge) for 5 min at 4°C to further remove extracellular bacteria. The supernatant containing the noningested bacteria were decanted, and the DC pellet was resuspended in 3 volumes of H buffer (8.6% sucrose, 25 mM HEPES, 5 mM MgCl₂, 1 mM PMSF), then homogenized with Dounce Homogenizer. Cells were observed under microscope to ensure lysis. The homogenate was centrifuged at 100 × g (1000 rpm on Eppendorf Centrifuge 5415 R) to bring down any intact cells and heavier debris. The supernatant containing phagosomes and lighter membranes were carefully layered on 62% sucrose, and centrifuged at 13,000 rpm for 45 min using Beckman Coulter Optima MAX ultracentrifuge (MLS-50 rotor). The lighter membranes remained on top of the 62% sucrose, while the heavier phagosomes were pelleted to the bottom. The phagosomal pellet was washed twice with ice-cold PBS and then processed for Western blot analysis or immunofluorescent staining.

Plasmid transfection and selection of stable cells

Transfection was performed either with Qiagen Effectene Reagent kit or Invitrogen Life Sciences Lipofectamine 2000, according to the manufacturer’s protocol. At 24–48 h after transfection, the cells were observed for transfection efficiency, after which it was diluted to an appropriate concentration. Geneticin (Invitrogen Life Technologies), otherwise known as G-418, was added at 2000 µg/ml to select for cells stably expressing VAMP-8.

Gene knockdown

Transfection was performed using Invitrogen Life Technologies Lipofectamine 2000. Small interfering RNA (siRNA) directed toward human VAMP-8 (catalog no. LQ-062971-01), as well as nontargeting siRNA pool (catalog no. D-001810-10), were purchased from Dharmacon. The amount of siRNA used for transfection was optimized at 2000 pmol per 10-cm dish transfection. The cells were assayed for their phagocytic activity at 48 h posttransfection.

Western blot analysis

Proteins resolved by SDS-PAGE were electroblotted to a nitrocellulose membrane (Hybond) and incubated overnight at 4°C with blocking buffer (PBS containing 5% skim milk and 0.05% Tween 20). Primary and secondary Abs incubations were done in blocking buffer. The membrane was washed with PBS containing 0.05% Tween 20 and then analyzed using the Supersignal Chemiluminescent kit (Pierce) according to the manufacturer’s recommendation.

FIGURE 2. VAMP-8 is recruited to both the early and late phagosomes, whereas VAMP-3 preferentially recruited to the early phagosomes upon phagocytosis of GFP-E. coli. A, Kinetic assay showing the recruitment of VAMP-8 to the phagosomes. Adherence of GFP-bacteria to cell surface was performed at 0°C (on ice) for 30 min. The time points tested were

5, 10, 15, 20, and 30 min and 1, 2, 3, and 4 h. Only selected time points were presented. Arrows point to selected VAMP-8-positive phagosomes. B, The cells were similarly stained for VAMP-3. Arrows indicate VAMP-3-positive phagosomes. C, RAW 264.7 cells were allowed to internalize GFP-E. coli for 1 h, fixed, and stained for VAMP-8. Arrows point to selected VAMP-8-positive phagosomes. Scale bar represents 10 µm.
VAMP-8 colocalizes with ingested GFP-\textit{E. coli}. 

\textbf{FIGURE 3.} VAMP-8 colocalizes with ingested GFP-\textit{E. coli}. \textit{A}, Stereo three-dimensional rendering of collected confocal images showing the recruitment of VAMP-8 (red) to the GFP-\textit{E. coli}-containing phagosomes (green) at 0-20 min at 37°C and 1-h time points. Arrows indicate colocalization between VAMP-8 and GFP-\textit{E. coli} phagosomes. Scale bar represents 5 \textmu m. \textit{B}, X-Z/Y-Z projection of collected confocal images at 0-h time point showing the colocalization of VAMP-8 and GFP-\textit{E. coli} at two different positions. Arrows indicate VAMP-8/GFP-\textit{E. coli}-positive phagosomes, and arrowheads indicate possible extracellular GFP-\textit{E. coli} bound to the surface of DC. Z-scaling = 0.43 \textmu m, Z-stack size = 19.34 \textmu m (n = 46 slices). \textit{C}, X-Z/Y-Z projection of collected confocal images at 1-h time point showing more intracellular VAMP-8/GFP-\textit{E. coli}-positive phagosomes, as indicated (arrows). Z-scaling = 0.43 \textmu m, Z-stack size = 14.18 \textmu m (n = 34 slices). Stereo three-dimensional rendering and X-Z/Y-Z projection were performed using the velocity visualization software from Improvision.

\textbf{Immunofluorescence microscopy}

Cells grown on coverslips were washed once with PBS, then fixed with 4% paraformaldehyde for 30 min on ice, after which they were washed thrice with PBS, followed by twice with 0.1% saponin in PBS. The cells were then incubated for 20 min in 0.1% saponin at room temperature to permeabilize them, after which primary Ab was then added and incubated for 1 h, followed by three washing steps with 0.1% saponin. Secondary Ab was then added and the coverslips were incubated in the dark for 1 h, washed four times with 0.1% saponin, and mounted on slides using VectaShield mounting medium with DAPI. Alternatively, cells were fixed with cold methanol at -20°C for 4 min and washed extensively with PBS before labeling them with Abs. Confocal images were taken using either Olympus Fluoview 500 microscope with Fluoview version 5.0 software or Zeiss LSM 510 Meta (upright stage) with Zeiss LSM Image Browser version 4.0.0.091 software. The conventional fluorescent images were taken using Olympus BX-60 digital microscope with ImagePro Plus software.

\textbf{Results}

\textbf{VAMP-8 and LAMP-2 colocalize in DC}

VAMP-8 proteins were previously shown to be preferentially enriched in the early endosomes (13), and late endosomes (15). To determine whether VAMP-8 colocalizes with LAMP-2, a late endosomes/lysosomes protein marker in DC, confocal immunofluorescent microscopy was performed using specific Abs against VAMP-8 and LAMP-2. VAMP-8 and LAMP-2 colocalized well in both the DC line, DC2.4 (Fig. 1, a and b) and bone marrow-derived DC (Fig. 1, e–g), indicating that the majority of VAMP-8 was localized to the late endosomal compartments in DC. In contrast, VAMP-3, an early/recycling endosomes localized v-SNARE, (3) did not colocalize with LAMP-2 in DC2.4 line (Fig. 1, c and d). It is worth noting that not all VAMP-8-positive endosomes colocalized with LAMP-2, indicating that at least a smaller pool of VAMP-8 was also localized to the early endosomes in DC.

\textbf{VAMP-8 is recruited to the early and late phagosomes upon exposure to bacteria}

Unlike VAMP-3 (3) and VAMP-7 (7), VAMP-8 has not been shown to be recruited to phagosomes. Because VAMP-8 was localized to both the early and late endosomes in DC, it is interesting to determine whether VAMP-8 was also recruited to the phagosomes during phagocytosis. DC were preincubated with GFP-\textit{E. coli} at 37°C for 20 min, after which they were washed to remove extracellular unbound GFP-\textit{E. coli}. Taking 37°C/20 min as the zero time point, the cells were allowed to internalize the GFP-\textit{E. coli} (green) for 5 h (a and b). Cells were fixed, labeled for VAMP-8 (red), and viewed under a confocal microscope. Small and round VAMP-8/GFP-\textit{E. coli}-positive phagosomes (yellow) were indicated with long arrows. Normal rod-shaped VAMP-8/GFP-\textit{E. coli} phagosomes (yellow) were indicated with short arrows. By incubating DC with unlabeled \textit{E. coli}, similar VAMP-8-positive structures that are also stained positively for LAMP-2 at 5 h post-ingestion were observed, further suggesting that VAMP-8 was indeed associated with the late-phagosomes/phagolysosomes (c–f). Three-dimensional rendering was performed using the velocity visualization software from improvision. Z-scaling = 0.43 \textmu m, Z-stack size = 19.77 \textmu m (n = 47 scales). Scale bar represents 10 \textmu m.
coli for 30 min on ice (0°C), washed, and allowed to internalize the GFP-E. coli at 37°C for various time points. As shown in Fig. 2A, VAMP-8 was recruited to the phagosomes as early as 10 min (Fig. 2A, a–c), and partially recycled off at the 2-h time point (Fig. 2A, m–o) as the early phagosomes underwent progressive maturation to become late phagosomes/phagolysosomes. However, only a small amount of VAMP-3 could be detected on the phagosomes even at the 1-h time point, suggesting that VAMP-3 preferentially recruited to the early phagosomes and VAMP-8 recruited to both the early and late phagosomes (Fig. 2B). Braun et al. (7) reported previously that VAMP-8 was not recruited to phagosomes during phagocytosis of IgG-opsonized sheep RBC in RAW 264.7 cells. To address this discrepancy, we allowed RAW 264.7 cells to internalize GFP-E. coli for 1 h, then cells were fixed and stained for VAMP-8 proteins. Like in DC, VAMP-8 was found to accumulate around the internalized GFP-E. coli (Fig. 2C), suggesting that phagosomal recruitment of VAMP-8 occurs during Fc receptor-independent phagocytosis.

To further confirm that VAMP-8 was recruited to the late phagosomes, we preincubated DC2.4 cells with GFP-E. coli for 20 min at 37°C (to enhance binding of GFP-E. coli to cells), then washed extensively to remove unbound bacteria. Taking that as the zero time point, the cells were allowed to internalize the GFP-E. coli for various time points. By using stereo three-dimensional rendering and X-Z/Y-Z projections of the collected stack confocal images, we further confirmed that a substantial amount of VAMP-8 was recruited to the phagosomes in DC (Fig. 3, open arrows and arrowheads). At 5 h postingestion of bacteria, most of the VAMP-8/GFP-E. coli-positive structures (presumably phagolysosomes) were smaller and rounder in shape as compared with those at the earlier time points (Fig. 4, a and b, long and short arrows). This finding could be due to degradation of GFP-E. coli in the phagolysosomes and the recycling of VAMP-8 to the early and late endosomes (at steady states). By incubating DC with unlabeled E. coli, we observed similar VAMP-8-positive structures that were also stained positive for LAMP-2 at 5 h postingestion, further suggesting that VAMP-8 was indeed associated with the late phagosomes/phagolysosomes (Fig. 4, c–f). To further confirm phagosomal recruitment of VAMP-8, GFP-E. coli-loaded phagosomes were purified from DC by sucrose gradient, lysed, and analyzed by Western blotting using Abs specific for VAMP-3 (positive control), VAMP-8, and Syn-16 (negative control). As expected, both VAMP-3 and VAMP-8, but not Syn-16 (a Golgi SNARE), were enriched in the phagosomal fraction (Fig. 5A). Immunofluorescent staining of the isolated phagosomes also revealed VAMP-3- and VAMP-8-positive GFP-E. coli-phagosomes (Fig. 5B).

VAMP-8 negatively regulates phagocytic activity in DC

Because a significant amount of VAMP-8 was recruited to the phagosomes, it is therefore interesting to determine whether VAMP-8 is required for phagocytosis in DC. DC phagocytic activity was measured using the Vybrant Phagocytosis Assay kit purchased from Molecular Probes. After normalizing the cell concentration, VAMP-8−/− and VAMP-8+/+ (wild type) bone marrow-derived DC were seeded into 96-well ELISA plates (Costar...
and incubated with fluorescence-conjugated E. coli bioparticles for 2 h. The unbound bioparticles were then removed and extracellular membrane-bound bioparticles were quenched with trypan blue. Fluorescence from engulfed bioparticles was then quantified using an ELISA fluorescence reader. Interestingly, the phagocytic activity of VAMP-8 was 48% higher as compared with that of VAMP-8 (Fig. 6A). The corresponding Western blot showed the presence of a 15-kDa band in VAMP-8 DC. No band was detected in VAMP-8 DC. Similarly, DC2.4 cells transfected with siRNA specific for VAMP-8 also exhibited enhanced phagocytic activity (Fig. 6B, a). In contrast, transfection with siRNA-specific VAMP-3 has no significant effect on phagocytosis. The corresponding Western blot confirmed the silencing of VAMP-8 and VAMP-3. When the cells were allowed to internalize GFP-E. coli for 1 h, it could even be visually seen that VAMP-8 knockdown cells exhibited enhanced phagocytosis (Fig. 6B, b). To determine whether maturation of phagosome is affected in VAMP-8 knock down cells, DC were allowed to internalize GFP-E. coli for 1 h, washed extensively to remove extracellular GFP-E. coli, then fixed and viewed under microscope at ×10 magnification (b). Enhanced phagocytosis was observed in VAMP-8 knockdown cells even at visual level. Scale bar represents 100 μm. C. DC2.4 cells were transfected using Lipofectamine 2000 with either scramble siRNA or siRNA directed toward VAMP-8. GFP-E. coli was efficiently silenced in almost all the cells (a). Scale bar represents 50 μm. The same batch of cells was allowed to internalize GFP-E. coli for 1 h. Silencing of VAMP-8 resulted in accelerated phagosome maturation (b). Arrows indicate round bacterial structures that were most likely phagolysosomes. Bar, 10 μm. D. DC2.4 cells triple-transfected with pDMyc, VAMP-3, and VAMP-8 were compared. Phagocytic activity in VAMP-3-transfected DC2.4 cells did not show significant difference with those transfected with pDMyc. Comparatively, phagocytosis was down-regulated by ~38% in VAMP-8-transfected DC2.4 cells. Immunofluorescent images and immunoblot confirmed successful overexpression of the proteins. Transfection was performed using Qiagen Effectene Transfection kit. Images were taken using Olympus BX-60 digital microscope at ×10 magnification. Scale bar represents 100 μm. E. The number of bioparticles ingested was manually counted under 100X immersion oil objective; n = 24 view areas randomly selected for counting; n = 95 VAMP-8-transfected cells counted; and n = 323 untransfected cells counted. The average number of bioparticles engulfed per cell was then computed and presented in terms of a percentage. The number of bioparticles engulfed by VAMP-8-transfected cells was reduced by >4-fold as compared with those by nontransfected cells. Experiment was repeated three times and similar trend was observed. Fluorescent reading was quantitated using SpectraMax Gemini EM ELISA plate reader (A and C) or Tecan GENios ELISA plate reader (B).
1 h. Silencing of VAMP-8 resulted in accelerated phagosome maturation as depicted from the round bacterial structures (phagolysosomes) present in VAMP-8 knock down cells but not in the control cells (Fig. 6C). As expected DC2.4 cells transiently expressing Myc-VAMP-8 showed a marked decrease in phagocytic activity as compared with control (Fig. 6D). The corresponding western blot showed the presence of an ~19-kDa band (Myc-VAMP-8) in cells transfected with VAMP-8. Consistent with previous findings that VAMP-3 was not required for phagocytosis (5, 6), our results showed that VAMP-3 did not cause a significant change in phagocytosis. The corresponding Western blot showed the expression level of both Myc-VAMP-3 and Myc-VAMP-8. Furthermore, phagocytosis assay performed on Myc-VAMP-8 stable clones also showed a drastic drop in phagocytic activity (data not shown).

To further confirm the phagocytic inhibition effect by VAMP-8 overexpression in DC, DC2.4 cells were transiently transfected with Myc-VAMP-8 and incubated with the *E. coli* bioparticles for 2 h. Extracellular membrane-bound bioparticles were quenched with trypan blue for 2 min and washed with PBS. Cells were then fixed with methanol and then labeled with anti-c-myc Ab (to detect Myc-VAMP-8) and secondary rhodamine-conjugated goat anti-mouse IgG. Under 100X oil immersion objective, the number of bioparticles in pDMyc-VAMP-8 and vector transfected cells were quantified. A total of 24 view areas were randomly selected for counting. The amount of bioparticles engulfed (or ingested) by

![FIGURE 7. VAMP-7 and VAMP-8 colocalize in the endosomes.](Image)

**A**, DC2.4 cells were transfected with pDMyc-VAMP-7, fixed, labeled with anti-c-myc (to detect Myc-VAMP-7) and anti-VAMP-8 Abs, and viewed and analyzed using a confocal imaging system. VAMP-8 (red) colocalizes with VAMP-7 (green) in the endosomes. VAMP-7/VAMP-8-positive endosomes were labeled yellow. **B**, Stereo three-dimensional rendering images of VAMP-8 and Myc-VAMP-7 subcellular localization in DC. Colocalization of VAMP-8 (red) with Myc-VAMP-7-positive endosomes (green) viewed at two different angles. VAMP-8/Myc-VAMP-7-positive endosomes were indicated with arrows. Z-scaling = 0.49 μm, Z-stack size = 12.26 μm (n = 26 slices). Transfection was performed using Lipofectamine 2000. Scale bar represents 10 μm.

![FIGURE 8. VAMP-7 inhibits phagocytosis in DC.](Image)

**A**, DC2.4 cells were transfected using Lipofectamine 2000. After 24 h, the cells were assayed for their phagocytic activities. Overexpression of VAMP-3 had no effect on phagocytosis, whereas overexpression of VAMP-7 and VAMP-8 down-regulated it. Phagocytic activity of cells cotransfected with VAMP-7 and VAMP-8 was further down-regulated. Corresponding immunoblots and immunofluorescent images indicate protein expression and transfection efficiency. Scale bar represents 100 μm. **B**, DC2.4 cells were transfected with pDMyc vector or pDMyc-VAMP-7. At 24 h posttransfection, the cells were allowed to internalize GFP-*E. coli*. Isolated phagosomes were processed for western blotting and probed for Myc-VAMP-7 and endogenous VAMP-8. The band intensity was quantified using ImageJ software, normalized to the same amount of protein, and represented as shown. The ratio of phagosomal extract to total lysate (PE:TL) was computed.
Myc-VAMP-8 transfected cells was drastically reduced as compared with control (Fig. 6E). Taken together, our results strongly suggest that VAMP-8 negatively regulates phagocytosis of bacteria in DC.

**VAMP-7 colocalizes with VAMP-8 and inhibits phagocytosis in DC**

Both VAMP-8 and VAMP-7 had been shown to interact with the Syn-7/Syn-8/Vti1b complex in the endosomes (18) and were reported to be essential for the membrane fusion events in the endosomes most probably via their ability to form complexes with Syn-7/Syn-8/Vti1b (18). There is a possibility that the phagocytosis inhibitory effects of VAMP-8 on phagocytosis in DC could be due to its ability to form complexes with Syn-7/Syn-8/Vti1b. VAMP-8/Syn-7/Syn-8/Vti1b complex formation might enhance membrane homotypic fusion events in the endosomes and indirectly affects the phagocytic process. If this is the case, it is interesting to determine whether overexpression of VAMP-7 (which can also form complexes with Syn-7/Syn-8/Vti1b and had been shown to be able to enhance membrane heterotypic fusion events in the endosomes) in DC can inhibit phagocytosis. To gain insight into this possibility, Myc-tagged VAMP-7 (Myc-VAMP-7) was overexpressed in DC. Confocal immunofluorescence microscopy showed that Myc-VAMP-7 colocalized well with VAMP-8 in the endosomal membranes of DC (Fig. 7). As anticipated, like in the case of VAMP-8 overexpression, overexpression of Myc-VAMP-7 but not Myc-VAMP-3 (as control) inhibited phagocytosis in DC (Fig. 8A). In addition, overexpression of both Myc-VAMP-7 and Myc-VAMP-8 further hindered the phagocytic activity (Fig. 8A). Interestingly, we also observed that overexpression of Myc-VAMP-7 significantly reduced recruitment of endogenous VAMP-8 to phagosomes (Fig. 8B), thus suggesting that VAMP-7 competed efficiently with VAMP-8 recruiting to phagosomes.

**Discussion**

Based on previous reports (the link between VAMP-3 and VAMP-7 and phagocytosis), and our observation that VAMP-8 is localized to the phagosomes, we speculated that VAMP-8 plays a role in phagocytosis. However, interestingly we observed an increase in the phagocytic activity of VAMP-8−/− as compared with VAMP-8+/+ (wild type) DC. Consistent with this finding, we also found that overexpression of VAMP-8 reduced phagocytic activity. Our study suggests that VAMP-8 inhibits phagocytosis in DC. However, the inhibition of phagocytosis by VAMP-8 is not due to a reduction of cell surface scavenger receptors (data not shown).

The formation of phagosomes requires targeted delivery of vesicles from the recycling endosomal compartment, contributing to the elongation of membranous pseudopods (3). Once internalized, the phagosomal membrane undergoes progressive maturation and recruits membrane proteins found in the early endosomes, the late endosomes and the lysosomes (22). Multiple members of the VAMP and syntaxin families have been identified and localized to specific membrane compartments along the secretory and endocytic pathways. In vitro studies have shown that phagosome-endosome and phagosome-lysosome fusions are NSF-dependent, thus indicating a requirement for SNARE proteins in the phagocytosis process (23, 24). Furthermore, phagosomes isolated at various time points have different fusion capability, suggesting that temporal changes in its membrane protein composition influence its fusion capacity. Thus, recruitment of multiple SNARE proteins to the phagosomes over time is essential in regulating fusion events during phagocytosis. Membrane trafficking and fusion involves not only interaction between cognate SNARE proteins on opposing membranes; tethering factors interact with SNARE proteins and facilitate SNARE complex assembly. Tethering factors typically form highly extended coiled-coil structures and enhance the efficiency of membrane trafficking by linking the opposing membranes (25). In addition, it is generally known that the switching of NSF between its ATP and ADP bound forms, via ATP hydrolysis, is required for SNARE complex disassembly.

SNAREs are initially functionally classified as vesicle-associated (v-SNAREs) and target compartment-associated (t-SNAREs) based on their vesicle or target membrane localization (26). SNAREs were later reclassified structurally as R-SNARE and Q-SNARE based on a highly conserved arginine and glutamine residue, respectively (27). Q-SNAREs are further divided based on the amino acid sequence homology of the SNARE domains. The syntaxin subfamily is also known as the Qa subfamily. SNAREs that are more homologous to the N-terminal of SNAP-25 are assigned the name S25N, otherwise known as Qb subfamily. Similarly, SNAREs that are more homologous to the C-terminal of SNAP-25 are assigned the name S25C, or Qc. It is believed that a SNARE complex is made up of one protein from each subfamily, that is, Qa:Qb:Qc:R.

VAMP-8, one of the SNARE types localized to the endosomes, had been shown to play important roles, from homotypic fusion of early and late endosomes (15, 18), exocytosis of vesicles (14, 19, 28, 29), cytokinesis (30), to its role in phagocytosis modulation (our current findings). VAMP-8 forms complexes with Syn-7, Syn-8, and Vti1b, which mediates homotypic fusion of early and late endosomes (15, 17, 18). VAMP-8 also forms complexes with Syn-4 and SNAP-23, which is involved in granule secretion of mast cells (28), granule secretion of platelets (29), and exocytosis of pancreatic acinar cells (19). Overexpression of VAMP-8 in MIN6 cells significantly increased GABA release (14), and although the precise complex involved was not defined, it is likely to involve the complex required for exocytosis. VAMP-8 has also been shown to be important in the terminal step of cytokinesis in the mammalian cells (30), where it was localized to the mid-body of late telophase NRK cells and that introduction of VAMP-8 lacking the C-terminal transmembrane region into MDCK cells resulted in the formation of binucleated cells. Although localized to the endosomes, recruitment of VAMP-8 to the phagosomes had not been reported before this. In this study, we reported that VAMP-8 was recruited to early and late phagosomes and it negatively regulates phagocytosis in DC. We showed that VAMP-8 proteins, presumably from the early endosomes were recruited to a small number of early phagosomes formed after 10 min incubation at 37°C (preincubation with GFP-E. coli at 0°C). At later incubation time points at 37°C, a higher number of VAMP-8-positive phagosomes were observed, indicating that VAMP-8 was preferentially recruited to the late phagosomes.

Recruitment of VAMP-8 to the late phagosomes was consistent with the fact that the majority of VAMP-8, which localized to the late endosomal compartments, colocalized with LAMP-2 in DC (in this study). Because VAMP-8 was recruited to the early and late phagosomes, it is therefore essential to determine whether VAMP-8 played important roles in the phagocytosis process. Interestingly, our results showed that VAMP-8 negatively regulates phagocytosis in DC. There is a possibility that VAMP-8 competes with another R-SNARE protein essential for phagosomes formation such as VAMP-3 and VAMP-7. This hypothesis is supported by the early recruitment of VAMP-8 to the phagosomes in DC (Fig. 3). Although VAMP-3 had been shown to be recruited to the phagosomes in macrophages, phagocytosis process in VAMP-3...
knockout cells was not affected (5, 6). Consistently, we showed that silencing of VAMP-3 using siRNA specific for VAMP-3 and overexpression of VAMP-3 did not affect phagocytosis in DC (Figs. 5B and 6B). VAMP-8 has been reported to form complex with Syn-7, Syn-8, and Vti1b, which mediates homotypic fusion of early and late endosomes (15, 17, 18). As reported previously, Syn-7 was recruited to phagosomes in macrophages (31). Interestingly, this same set of Syn-7-containing Q-SNAREs (Syn-7-Syn-8-Vti1b) also partners with VAMP-7 for mediating heterotypic fusion of the late endosomes and lysosomes (18). VAMP-7 had been shown to play important roles in optimal phagocytosis of opsonized particles in macrophages (7). However, we observed that overexpression of VAMP-7 inhibited phagocytosis in DC in this study.

Because both VAMP-8 and VAMP-7 had been reported to interact with a common SNARE partner (Syn-7-Syn-8-Vti1b complex), and VAMP-7 and VAMP-8 colocalized on the endosomal membranes in DC (in this study), it is possible that VAMP-7 could compete with VAMP-8 (or vice versa) during recruitment to the phagosomal membranes. As expected, we observed that overexpression of Myc-VAMP-7 reduced the recruitment of endogenous VAMP-8 to the phagosomal membranes, suggesting that there were significant competitive interactions of VAMP-8 and VAMP-7 with Q-SNAREs on phagosomes. However, because coexpression of both Myc-VAMP-7 and Myc-VAMP-8 in DC can further inhibit phagocytosis, there is also a possibility that VAMP-7 and VAMP-8 might form partners with different sets of Q-SNAREs on the phagosomal membranes. In this context, VAMP-7 and VAMP-8 might compete with another unknown but essential SNARE-like protein(s) for the same set(s) of Q-SNAREs. Further study along this line will lead us to identify various Q-SNARE complexes that might enhance phagosomes maturation and phagocytosis.

Further study along this line will lead us to identify various Q-SNARE complexes that might enhance phagosomes maturation and phagocytosis. Thus, functional interactions between endosomal SNAREs create a “balancing act” in the regulation of phagocytosis in phagocytic cells.

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Disclosures
The authors have no financial conflict of interest.

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