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Caspases regulate VAMP-8 expression and phagocytosis in dendritic cells

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ABSTRACT

During an inflammation and upon encountering pathogens, immature dendritic cells (DC) undergo a maturation process to become highly efficient in presenting antigens. This transition from immature to mature state is accompanied by various physiological, functional and morphological changes including reduction of caspase activity and inhibition of phagocytosis in the mature DC. Caspases are cysteine proteases which play essential roles in apoptosis, necrosis and inflammation. Here, we demonstrate that VAMP-8, (a SNARE protein of the early/late endosomes) which has been shown previously to inhibit phagocytosis in DC, is a substrate of caspases. Furthermore, we identified two putative conserved caspase recognition/cleavage sites on the VAMP-8 protein. Consistent with the up-regulation of VAMP-8 expression upon treatment with caspase inhibitor (CI), immature DC treated with CI exhibits lower phagocytosis activity. Thus, our results highlight the role of caspases in regulating VAMP-8 expression and subsequently phagocytosis during maturation of DC.

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Introduction

Caspases are a large family of cysteine proteases that uses cysteine as the neucleophilic group to cleave substrate at the c terminus of aspartic acid [1,2]. Caspases were initially extensively characterized based on their function in programmed cell death or apoptosis. However, mammalian caspases have also evolved additional roles in inflammatory responses [2]. In this context, caspases have been shown to play essential role in the activation of T, B and Natural Killer (NK) cells [3–7]. Furthermore, caspases are known to depresss cardiac function by their action in cardiac myocytes [8]. More recently, a novel role of caspases in regulating the expression of several proteins potentially important in governing the trafficking of major histocompatibility class II (MHCII) during dendritic cell (DC) maturation, was demonstrated [9]. We hypothesized that caspases could play a role in controlling the phagocytosis process which is also regulated by the endosomal membrane trafficking pathway, in DC.

Immature DC are characterized by their highly phagocytic activity. Upon encountering pathogens and subsequent internalization, these immature DC undergo a maturation process to become highly efficient in presenting antigens, concomitantly losing their phagocytic capability. This transition from immature to mature state is accompanied by many physiological, functional and morphological changes. The ability of dendritic cells, macrophages and neutrophils, to engulf and destroy invading microorganisms, serves to protect the host against a wide variety of infections. The ingestion of foreign particles, termed phagocytosis, involves a well-orchestrated cascade of events. Receptor-mediated binding of microorganism to the surface of phagocytic cell triggers Rac1- and Cdc42-dependent actin polymerization near the phagocytosis site, forming the membranous pseudopodia extension which engulfs and seals the microorganism in a vacuole-like structure called the phagosome [10]. Previous reports suggested that the uptake of antigens via phagocytosis was perturbed during maturation of DC [11] and that the endosomal soluble N-ethylmaleimide sensitive factor (NSF) attachment receptor (SNARE) VAMP-8 proteins has a role to play in regulating the phagocytic activity [12]. Here, we demonstrate the role of caspases in regulating the expression of VAMP-8 and the phagocytosis process in the maturing DC.

Materials and methods

Antibodies, enzymes and chemicals. The following antibodies were purchased commercially: VAMP-3 (Synaptic Systems), VAMP-8 (Synaptic Systems), CD74 (BD Biosciences Pharmingen), polyclonal anti-myc (Santa Cruz), β-actin (Sigma–Aldrich), fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), and HRP-conjugated antibodies (Pierce). The restriction endonucleases EcoRI and XbaI, T4 DNA ligase and *Pfu* polymerase were purchased from Promega. Caspase inhibitor I and spermine NONOate were from Calbiochem.

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Cloning of VAMP-8. VAMP-8 was cloned as previously described [12].

Site-directed mutagenesis of VAMP-8 gene. The D11A, D50A, and D11A/D50A mutants of VAMP-8 was generated by PCR mutagenesis using primers: 5′-ggaattccatggaggaggccagtgggagtgccggaaatgc ccg-3′, 5′- aagacagaggccttggaagccacgtctgaa-3′, 5′- ggcttccaaggcct ctgtcttgtttcggag-3′ and cloned into pDMyc vector using EcoRI (5′) and XbaI (3′) and confirmed via sequencing. Transfection was performed with Invitrogen Lipofectamine™ 2000, according to the manufacturer's protocol.

Preparation of primary dendritic cells. Primary mouse bone marrow-derived dendritic cells were prepared according to method reported previously [9,12,13].

Phagocytosis assay. The phagocytosis assay is based on the Vybrant® Phagocytosis Assay Kit, with slight modification [12]. Briefly, cells seeded into a 96-well tissue culture plate at a concentration of $1–2 \times 10^5$ cells/well were allowed to adhere for 4 h to the bottom of the well, after which the fluorescence-conjugated *Escherichia 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. Trypan Blue was then removed and the amount of bioparticles engulfed by the cells was quantitatively measured using either SpectraMax Gemini EM or Tecan Infinite M200 plate reader at 480 nm excitation and 520 nm emission.

Transfection. Transfection was performed using Invitrogen Lipofectamine™ 2000. The cells were used 48 h post-transfection.

Immunofluorescence microscopy. Cells grown on cover slips 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 permeabilise them, after which primary antibody was then added and incubated for 1 h, followed by three washing steps with 0.1% saponin. Secondary antibody was then added and the cover slips 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 grown on cover slips were washed once with PBS, then fixed with cold methanol at -20 °C for 4 min and washed extensively with PBS before labeling them with antibodies. Images were captured using Olympus BX-60 digital microscope/ImagePro Plus software.

Statistics. Student's *t* test was used for statistical analysis.

Results

VAMP-8 is a substrate for caspases and is up-regulated during LPS-induced DC maturation

Garrett et al. [11] reported previously that the uptake of antigens via phagocytosis and macropinocytosis was inhibited during DC maturation. Consistently, we also observed a drastic down-regulation of phagocytosis in the lipopolysaccharide (LPS)-induced mature DC in our laboratory (Fig. 1B). Previously, VAMP-8 [14]

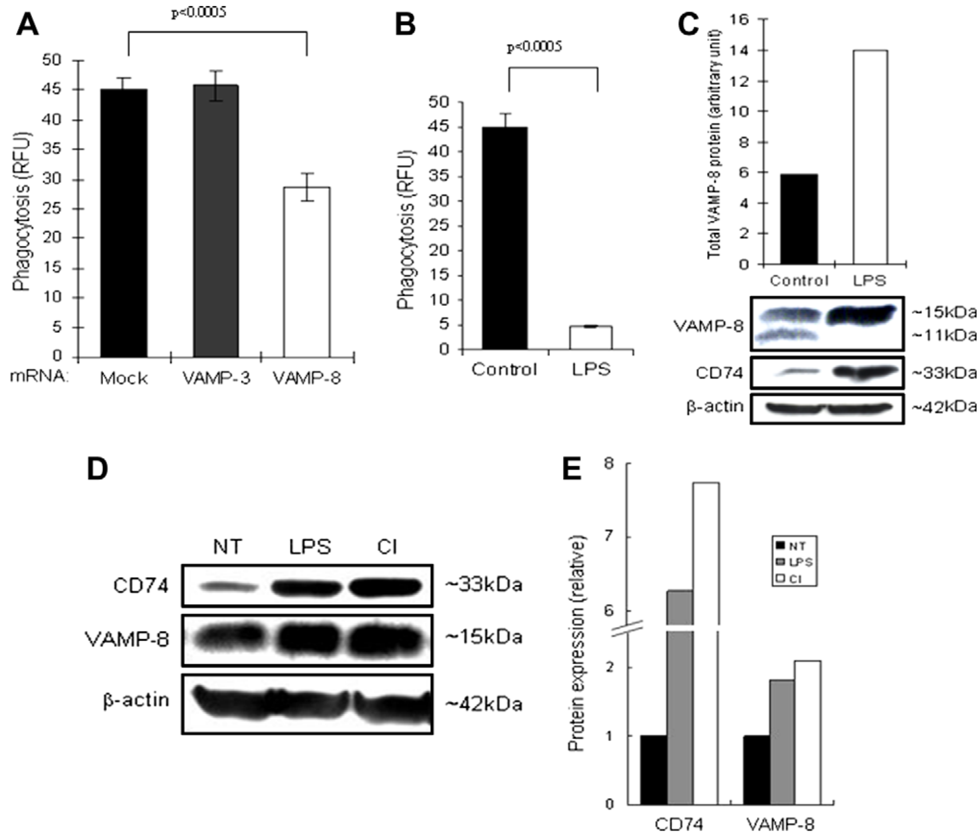

Fig. 1. Inhibition of phagocytosis during DC maturation is concerted with an up-regulation of VAMP-8 protein expression. (A) Transfection (electroporation) of *in vitro*-transcribed VAMP-8 mRNA into immature primary DC inhibited phagocytosis. Immature primary DC transfected with VAMP-3 mRNA was used as negative control. Data shown are the mean (SD) of triplicate samples and are representative of three independent experiments with similar results. Stastically significant differences (*p*-value) are as indicated. (B) LPS (1 μg/ml) treatment of primary DC for 24 h inhibited phagocytosis in primary DC. (C) The same batch of untreated and LPS-treated primary DC from "*B*" were lysed and analysed by SDS–PAGE and Western blot using antibodies specific for CD74 (Ii), VAMP-8, and β-actin. VAMP-8 protein was up-regulated by LPS in primary DC. CD74 was used to indicate DC maturation. The band intensities were quantified with ImageJ software, normalized with β-actin and presented as a bar chart. Data shown are representative of at least three independent experiments with similar results. (D) Treatment of immature primary DC with 100 μM caspase inhibitors for 4 h up-regulates VAMP-8 protein expression as detected by Western blot using antibodies specific for VAMP-8, CD74 and β-actin. (E) The band intensities in "*D*" were quantified with ImageJ software, normalized with β-actin and presented as a bar chart. Data shown are representative of at least three independent experiments with similar results.

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