Acid sphingomyelinase is required for efficient phago-lysosomal fusion

I n a u g u r a l – D i s s e r t a t i o n

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln 2008

Tag der letzten mündlichen Prüfung: 25.11.08

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Abbreviations

ASMase	Acid sphingomyelinase
ASMase$^{-/-}$	Acid sphingomyelinase-deficient
BHI	Brain Heart Infusion
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
CFU	Colony forming unit(s)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EEA1	Early endosomal antigen 1
Fig.	Figure
FITC	Fluorescein isothiocyanate
H$^+$	Proton
HBSS	Hanks Balanced Salt Solution
HKLM	Heat-killed *Listeria monocytogenes*
i.p.	intra peritoneal
IFM	Immunofluorescence microscopy
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
iNOS	Inducible nitric oxide synthase
kDa	kiloDalton
L.m.	*Listeria monocytogenes*
Lamp1	Lysosome-associated membrane protein 1
LLO	Listeriolysin O
M6PR	Cation-independent mannose 6-phosphate receptor
MACS	Magnetic cell sorting
MEF	Mouse embryonic fibroblasts
MOI	Multiplicity of infection
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate

NMS	Normal mouse serum
NO	Nitric oxide
NPD	Niemann-Pick disease
O_2	molecular oxygen
O_2^-	Superoxide
OD_{600}	Optical density at 600 nm
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
pH	pH value; negative decadic logarithm of the H^+ concentration
Phox	Phagocyte oxidase
PMA	Phorbol 12-myristate 13-acetate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temparature
s.e.	Standard error of the mean
SDS	Sodium dodecyl sulfate
SMase	Sphingomyelinase
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SV	Simian virus
TNF	Tumour necrosis factor
TRITC	Tetramethylrhodamine isothiocyanate
vATPase	vesicular proton pump
w/v	Weight to volume
WB	Western blot
wt	Wild type

1 Introduction

1.1 Acid sphingomyelinase

Sphingomyelinases (SMases) catalyze the hydrolysis of the membrane lipid sphingomyelin into ceramide and phosphorylcholine. So far, seven sphingomyelinases have been identified and classified with regard to pH optimum, subcellular localization or dependency on bivalent metal ions. The most intensively studied SMase is the acid sphingomyelinase (ASMase) (Levade and Jaffrezou 1999).

ASMase is expressed ubiquitously in mammals but confined to a few subcellular locations, i.e. the extracellular leaflet of the plasma membrane, endosomes, phagosomes and lysosomes. Activity of ASMase is maximal at acidic pH of about 4.5 to 5 (Kanfer, Young et al. 1966; Schuchman, Suchi et al. 1991; Schissel, Schuchman et al. 1996; Schissel, Keesler et al. 1998; Kronke 1999; Grassme, Schwarz et al. 2001; Schneider-Brachert, Tchikov et al. 2004).

Deficiency in ASMase in human leads to autosomal recessive inherited Niemann-Pick disease (NPD) (Brady, Kanfer et al. 1966; Schneider and Kennedy 1967). NPD is a lipid storage disorder caused by abolished (type A NPD) or strongly reduced (type B NPD) activity of ASMase. Intracellular accumulation of sphingomyelin in neuronal cells of the cerebellum leads to severe neurodegeneration and death during early childhood (type A NPD) or adolescence (type B NPD). ASMase-deficient (ASMase$^{-/-}$) mice show first clinical symptoms of NPD at an age of about three months and die at about nine months of age (Horinouchi, Erlich et al. 1995; Lozano, Morales et al. 2001).

By hydrolyzing sphingomyelin, SMases alter the lipid composition and thereby the biochemical and biophysical properties of biomembranes. Sphingomyelin is an important component of the extracellular leaflet of biomembranes (Futerman and Riezman 2005; Holthuis and Levine 2005) and interacts with cholesterol to form lipid rafts which are central for the spatial organization of integral membrane proteins. Hydrolysis of sphingomyelin into ceramide alters lipid raft composition as ceramide spontaneously self-associates into ceramide-enriched membrane domains (Holopainen, Subramanian et al. 1998). Removal of the large polar head group phosphorylcholine from sphingomyelin also promotes transition from lamellar phase

into hexagonal II phase which facilitates membrane reorganization processes (Kronke 1999). Furthermore, ceramide is an important signalling molecule involved in programmed cell death, cell differentiation and proliferation (Futerman and Hannun 2004). By hydrolyzing sphingomyelin into ceramide SMases also directly influence the fusogenicity of biomembranes (Grassme, Gulbins et al. 1997; Zha, Pierini et al. 1998; Goni and Alonso 2000; Holopainen, Angelova et al. 2000; Goni and Alonso 2002) and promote liposome fusion *in vitro* (Ruiz-Arguello, Basanez et al. 1996; Basanez, Ruiz-Arguello et al. 1997; Hinkovska-Galcheva, Boxer et al. 1998; Oorni, Hakala et al. 1998; Ruiz-Arguello, Goni et al. 1998).

ASMase is activated by a number of pro-inflammatory cytokines, e.g. tumour necrosis factor (TNF), interleukin (IL)-1ß or interferon-γ (IFN-γ) suggesting a role of ASMase in immune responses (Schutze, Potthoff et al. 1992; Cifone, Roncaioli et al. 1995; Pushkareva, Obeid et al. 1995; Kronke 1999; Schneider-Brachert, Tchikov et al. 2004). Furthermore, NPD patients are more susceptible to respiratory diseases indicating a role of ASMase during infection (Jonas 1966; Dechovitz and Moffet 1968). Importantly, our group has shown that ASMase$^{-/-}$ mice are highly susceptible to infection with the facultative intracellular bacteria *Listeria monocytogenes* (*L. monocytogenes*) and *Salmonella typhimurium* because ASMase$^{-/-}$ macrophages are unable to restrict intracellular growth of these bacteria (Utermohlen, Karow et al. 2003). The precise antibacterial mechanism of ASMase, however, remains elusive.

1.2   Antibacterial effector mechanisms of macrophages

Macrophages are of critical importance in innate immunity as they specialize in phagocytosis and subsequent killing of invading bacteria. To inactivate and degrade phagocytosed bacteria, macrophages employ both oxidative and non-oxidative effector mechanisms (Scott, Botelho et al. 2003; Haas 2007).

1.2.1   Oxidative effector mechanisms

Macrophages generate oxidative effector molecules by two distinct pathways (Vazquez-Torres, Jones-Carson et al. 2000). First, the nicotinamide adenine dinucleotide phosphate-oxidase complex (NADPH oxidase complex; phox) generates reactive oxygen intermediates (ROI). Second, the inducible nitric oxide synthase (iNOS) generates reactive nitrogen intermediates (RNI).

The NADPH oxidase complex is assembled from its subunits in the membrane of phagosomes containing bacteria (Nauseef 2004; Minakami and Sumimotoa 2006). The catalytic subunit, flavocytochrome b_{558}, is a heterodimer composed of $gp91^{phox}$ and $p22^{phox}$. The flavocytochrome b_{558} is an integral membrane protein of the plasma membrane and of vesicles fusing with phagosomes very early after generation of phagosomes (Ginsel, Onderwater et al. 1990; Nauseef 2004). The regulatory subunits $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac2 are recruited to flavocytochrome b_{558} from their location in the cytosol (DeLeo, Allen et al. 1999; van Bruggen, Anthony et al. 2004). After assembly, the NADPH oxidase complex catalyses the transfer of one electron from NADPH through the phagosomal membrane onto molecular oxygen (O_2) which leads to generation of superoxide (O_2^-) inside phagosomes. The highly reactive radical superoxide reacts with intraphagosomal molecules which gives rise to diverse bactericidal ROI, e.g. hydrogen peroxide, hydroxyl radicals or hypochloride (Quinn and Gauss 2004; Robinson, Ohira et al. 2004).

Cytosolic iNOS is recruited to phagosomes containing bacteria where its homodimeric form catalyses the oxidation of L-arginine into L-citrulline and nitric oxide (NO) (Vodovotz, Russell et al. 1995; Miller, Fratti et al. 2004). The lipid and water permeable radical NO diffuses into the phagosome where it reacts with oxygen-containing molecules leading to generation of diverse bactericidal RNI, e.g. nitrogen dioxide, nitrite, nitrate or S-nitrothioles (MacMicking, Xie et al. 1997). After activation of macrophages, expression of iNOS is strongly up-regulated while resting macrophages express almost no iNOS (Aktan 2004).

The simultaneous generation of ROI and RNI has a synergistic effect on killing of phagocytosed bacteria because O_2^- and NO form the highly bactericidal peroxynitrite ($ONOO^-$) (Nathan and Shiloh 2000).

1.2.2 Non-oxidative effector mechanisms

For many years, ROI and RNI have been assumed to be the major antibacterial effector mechanisms (Klebanoff 1975; Nathan and Shiloh 2000). Only recently, it has been established that non-oxidative effector mechanisms, particularly lysosomal acid hydrolases, are at least as important for effective control of infectious pathogens within phagosomes (Segal 2005). Neutrophil elastase and cathepsin G are necessary for effective killing of S*taphylococcus aureus* and *Candida albicans* in neutrophils (Reeves, Lu et al. 2002). Furthermore, the lysosomal aspartic protease cathepsin D

has been identified as an important factor in the defence against *Listeria monocytogenes* in macrophages as well as in fibroblasts (del Cerro-Vadillo, Madrazo-Toca et al. 2006). The bactericidal acid hydrolases are transferred from lysosomes into phagosomes by phago-lysosomal fusion at the end of the phagosomal maturation process (Haas 2007).

1.3 Phagosome maturation

Phagosome maturation into phagolysosomes is achieved stepwise by sequential fusion of the phagosome with early endosomes, late endosomes and finally lysosomes (Mayorga, Bertini et al. 1991; Pitt, Mayorga et al. 1992; Desjardins, Celis et al. 1994; Desjardins, Huber et al. 1994; Desjardins, Nzala et al. 1997; Henry, Hoppe et al. 2004). Each maturation stage is characterized by acquisition or loss of specific marker molecules (Fig. 1). After separation from the plasma membrane, phagosomes fuse with early endosomes and by this acquire early endosomal markers like the 'early endosomal antigen 1' (EEA1). Via fusion with late endosomes, phagosomes acquire the 'cation-independent mannose 6-phosphate receptor' (M6PR) and the small GTPase Rab7. Only late phagosomes are capable of fusing with lysosomes into phagolysosomes. Phago-lysosomal fusion leads to transfer of mature acid hydrolases like the lysosomal cathepsins D, B and L from lysosomes into phagosomes. Furthermore, phagolysosomes are positive for the 'lysosome-associated membrane protein 1' (Lamp1) but lack M6PR and Rab7. With progressing phagosomal maturation, activity of the vesicular proton pump (vATPase) leads to a drop of the intraphagosomal pH from about 6.5 in early phagosomes to between 6.0 and 5.0 in late phagosomes and finally 5.0 to 4.0 in phagolysosomes.

Several molecules orchestrating the tethering, docking and final membrane fusion of endosomes or lysosomes with phagosomes have been identified in recent years (Luzio, Pryor et al. 2005). Key regulators of the phago-endosomal fusion machinery are Rab GTPases which recruit specific sets of effector proteins to vesicular membranes (Jordens, Marsman et al. 2005). After docking and tethering, membrane fusion itself is mediated by 'soluble N-ethylmaleimide-sensitive factor attachment receptor proteins' (SNAREs) (Jahn and Scheller 2006). In reconstituted systems *in vitro*, SNAREs have been shown to provide the minimal machinery for membrane
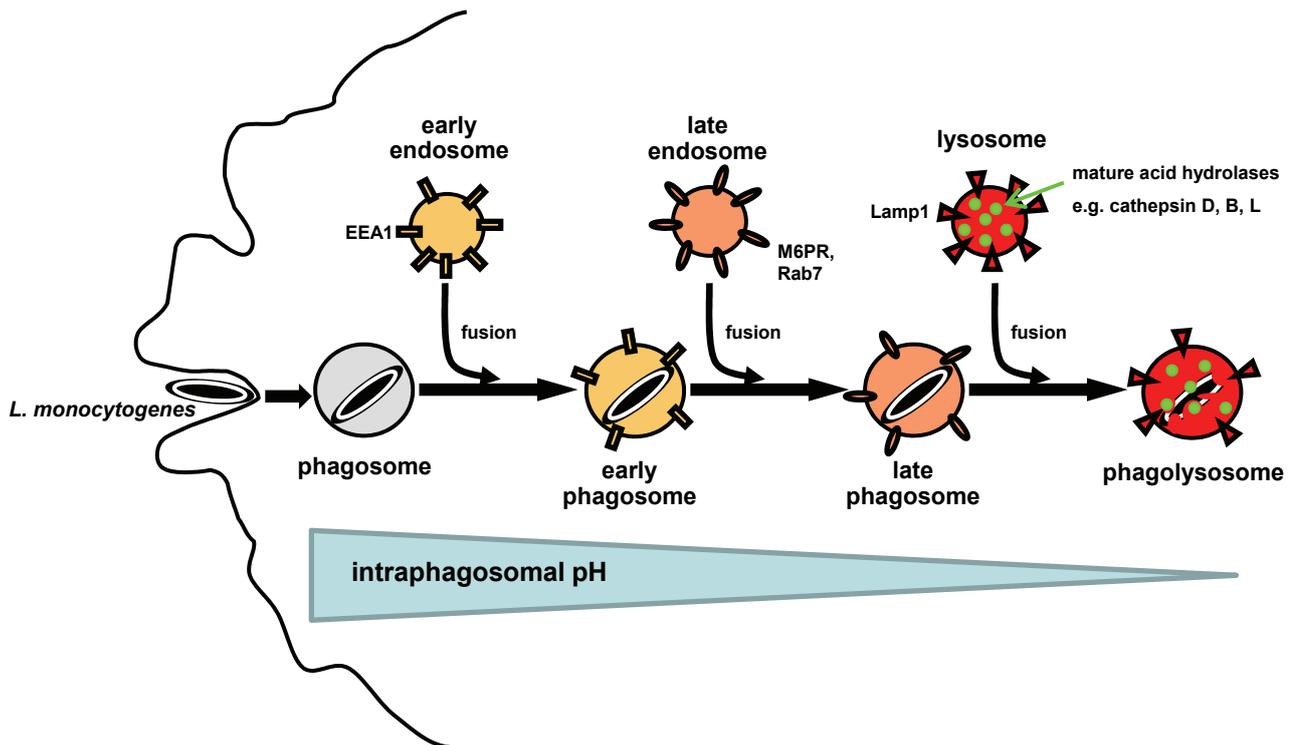
Fig. 1: Schematic overview of phagosomal maturation into phagolysosomes by sequential fusion with early endosomes, late endosomes and finally lysosomes.

fusion (Weber, Zemelman et al. 1998; Hu, Ahmed et al. 2003; Bonifacino and Glick 2004). However, this minimal fusion machinery acts very slowly and inefficiently so that additional factors are supposed to be involved in the rapid and efficient membrane fusion processes observed in intact, viable cells (Weber, Zemelman et al. 1998; Bonifacino and Glick 2004). Specifically, it has been hypothesized that lipid modifying enzymes might contribute to efficient vesicle fusion via alteration of the biochemical and/or biophysical properties of cellular membranes (Chernomordik and Kozlov 2003; Bonifacino and Glick 2004).

The kinetics of the phagosomal maturation and subsequent phago-lysosomal fusion are particularly critical during phagocytosis of intracellular bacteria which subvert phagosome maturation for their own needs (Scott, Botelho et al. 2003; Haas 2007). Some bacteria like for example *Mycobacterium tuberculosis* or *Salmonella typhimurium* arrest phagosome maturation at pre-phagolysosomal stage and thus avoid exposure to lysosomal acid hydrolases. Other bacteria like *Shigella flexneri* or *L. monocytogenes* disrupt the membrane of maturing phagosomes before phago-lysosomal fusion and escape into the cytosol.

1.4 Listeria monocytogenes

L. monocytogenes are a gram-positive rods causing severe, often lethal food-borne infections in immunocompromised individuals, pregnant women and newborns. The facultative intracellular bacteria cross the intestinal barrier by invading intestinal epithelial cells and primarily infect hepatocytes. For elimination of *L. monocytogenes* during primary infection, concerted action of almost all regulatory and effector mechanisms of innate and adaptive immunity is necessary (Gellin and Broome 1989).

For both pathogenesis and elimination, phagocytosis of *L. monocytogenes* by macrophages plays a crucial role. In resident macrophages, *L. monocytogenes* escape from phagosomes into the cytosol at about 30 min after infection (Myers, Tsang et al. 2003) and then proliferate with a generation time of about 1 h (Portnoy, Jacks et al. 1988). By disrupting the membrane of maturing phagosomes prior to phago-lysosomal fusion, *L. monocytogenes* avoid exposure to lysosomal acid hydrolases and at the same time gain access to the nutrient-rich cytosol. Three virulence factors are crucial for membrane disruption: the pore forming hemolysin listeriolysin O (LLO) (Leimeister-Wachter, Haffner et al. 1990; Beauregard, Lee et al. 1997) and the phospholipases PlcA and PlcB (Portnoy, Smith et al. 1994; Portnoy, Auerbuch et al. 2002). In the cytosol, *L. monocytogenes* recruit actin via its virulence factor ActA. The cloud of actin molecules surrounding *L. monocytogenes* is then rearranged into a comet-tail like structure. Continuous polymerization of actin at one bacterial pole propels *L. monocytogenes* towards the plasma membrane. There, *L. monocytogenes* induce formation of a pseudopod-like structure which is phagocytosed by neighbouring cells. *L. monocytogenes* disrupt the double membrane of the phagosome and enter the cytosol of the new host cell (Portnoy, Smith et al. 1994).

In contrast to resident macrophages, activated macrophages efficiently kill phagocytosed *L. monocytogenes*. Generation of large quantities of ROI and RNI (Dinauer, Deck et al. 1997; Shiloh, MacMicking et al. 1999; Alvarez-Dominguez, Carrasco-Marin et al. 2000; Myers, Tsang et al. 2003) and exposure to lysosomal acid hydrolases (del Cerro-Vadillo, Madrazo-Toca et al. 2006) prevent escape into the cytosol and lead to degradation of *L. monocytogenes* in phagolysosomes.

1.5 Aim of this study

ASMase$^{-/-}$ macrophages cannot kill phagocytosed *L. monocytogenes* despite activation (Utermohlen, Karow et al. 2003) indicating a role of ASMase in listeriocidal activity of macrophages. The aim of this study is to elucidate the listeriocidal mechanism of ASMase in macrophages.

2 Material and Methods

2.1 Material

2.1.1 Mice

Breeding pairs of mice heterozygously deficient for acid sphingomyelinase were kindly provided by R. Kolesnick (Memorial Sloan-Kettering Cancer Center, New York, NY; originally obtained from E. H. Schuchmann, Mount Sinai School of Medicine, New York, NY (Horinouchi, Erlich et al. 1995)). Mice were heterozygously backcrossed to the C57BL/6 strain to the 10th generation under specific pathogen-free conditions at the animal facilities of the Medical Centre of the University of Cologne (Cologne, Germany). Experiments were performed in accordance with the Animal Protection Law of Germany in compliance with the Ethics Committee at the University of Cologne with 6–10 weeks old ASMase$^{-/-}$ mice and wt littermates.

2.1.2 Bacteria

L. monocytogenes, strain EGD, serotype 1/2a, were kindly provided by C. Kocks (Harvard Medical School, Boston, USA). Following *in vivo* passage, single colonies of *L. monocytogenes* were expanded in brain-heart infusion (BHI) medium. Aliquots of log-phase growing cultures were stored at -80° C.

The isogenic Δ*prfA*- and Δ*hly*-deletion mutants of *L. monocytogenes* (Peters, Domann et al. 2003) were kindly provided by Eugen Domann (University of Giessen, Germany).

2.1.3 Chemicals, buffers and solutions

Chemicals were of research grade and from Sigma-Aldrich (Steinhausen, Germany), AppliChem (Darmstadt, Germany) or Becton Dickinson GmbH (Heidelberg, Germany) unless stated otherwise. Buffers and solutions were prepared using bidestilled H_2O from an EASYpure UV/UF H_2O purification unit (Werner Reinstwassersysteme, Leverkusen), degassed and sterilised by autoclaving or filtration through a 0.2 μm filter membrane if necessary.

0.1% saponin in PBS	0.1% (w/v) saponin in PBS, stored at 4° C
3 kDa dextran conjugated to TexasRed	Dextran, Texas Red, 3000 MW, lysine fixable (Invitrogen, Karlsruhe, Germany), stored at -20° C
3% PFA in PBS	3% paraformaldehyde in PBS, stored at -20° C
70 kDa dextran conjugated to TexasRed	Dextran, Texas Red, 70000 MW, lysine fixable (Invitrogen, Karlsruhe, Germany), stored at -20° C
Alexa Fluor 594-succinimidyl ester	5 mg/ml in DMSO (Invitrogen, Karlsruhe, Germany), stored at -20° C
Amersham ECL hyperfilm	GE Healthcare (München, Germany), stored at 4° C
BHI medium	Brain Heart Infusion medium
Blocking buffer (for IFM)	3% BSA, 0.1% saponin in PBS, stored at 4° C
Blocking buffer (for WB)	5 % non-fat dried milk powder in TBS-T, stored at -20° C
BSA	Albumin from bovine serum, fraction V, stored at 4° C
Carboxylated 1 µm silica beads	Silica particles carboxylated, 1,0 µm, 50 mg/ml (Kisker Biotech, Steinfurt, Germany), stored at 4° C
CD11b MicroBeads	CD11b MicroBeads, human and mouse (Miltenyi Biotec, Bergisch Gladbach, Germany), stored at 4° C
Chicken ovalbumin conjugated to TexasRed	Ovalbumin, Texas Red conjugate (Invitrogen, Karlsruhe, Germany), stored at -20° C
DMEM	1x Dulbecco`s Modified Eagle Medium, stored at 4° C
DMSO	Dimethyl sulfoxide (Merck, Darmstadt, Germany)
Ferricytochrome c	0,27 g cytochrome c from equine heart, type VI, in HBSS with Ca^{2+} and Mg^{2+}, stored at -20° C
Fetal calf serum (FCS)	heat-inactivated at 56° C for 30 min

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	(Biochrom AG, Berlin, Germany), stored at 4° C
FITC	Fluorescein isothiocyanate isomer I, 20 mg/ml in DMSO, stored at -20° C
Greiss reagent	1% (w/v) sulfanilamide in 2,5% phosphoric acid and 1% (w/v) naphthyl ethylene diamine dihydrochloride in 2,5% phosphoric acid, stored separately at 4° C
HBSS	"Hanks Balanced Salt Solution" with Ca^{2+} and Mg^{2+}
HEPES	1 M HEPES solution (Invitrogen, Karlsruhe, Germany), stored at 4° C
Human IgG	IgG from human serum, stored at -20° C
Immobilon-P^{SQ} transfer membrane	Millipore (Bedford, USA)
Interferon-γ (IFN-γ)	10 µg/ml interferon-γ from mouse in H_2O (R&D Systems, Wiesbaden-Nordenstadt, Germany), stored at -20° C
Laemmli buffer (5x)	60 mM Tris-HCl (pH 6.8), 2% SDS solution, 25% Glycerol, 0.2% bromphenol blue in H_2O, stored at -20° C; 10% ß-mercaptoethanol added before use
Lysis buffer	1% Triton X-100, 5 mM $MgCl_2$, 20 mM Tris-HCl in H_2O, pH 7.5
MES buffer	2 mM NaCl, 115 mM KCl, 1,2 mM $MgSO_4$, 25 mM MES in H_2O, pH 7.5; For calibration: adjusted to pH 3.0-7.5, stored at 4° C
Monensin	0.1 M monensin sodium salt in ethanol, stored at -20° C
MS columns	MS MACS Separation Columns (Miltenyi Biotec, Bergisch Gladbach, Germany), stored at 4° C
Nigericin	Nigericin sodium salt from *Streptomyces hygroscorpicus*, 50 mg/ml in chloroform, stored at -20° C
NuPAGE Novex 10% Bis-Tris Midi gels	Invitrogen (Karlsruhe, Germany), stored at 4° C
Oregon Green 488	Oregon Green 488-X succinimidyl ester, 6-

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	isomer (Invitrogen, Karlsruhe, Germany), 25 mM in DMSO, stored at -20° C
PBS	1 x Dulbecco´s phosphate buffered salt solution, pH 7.4 (Biochrom AG, Berlin), stored at 4° C
Penicillin/Streptomycin	Penicillin (10000 U/ml) and streptomycin (10 ng/ml) in H_2O (Biochrom AG, Berlin, Germany), stored at -20° C
Peptide substrate	(Biotin-LC-Phe-Arg)$_2$-rhodamine 110 (AnaSpec Inc., San Jose, USA), stored at -20° C
Phalloidin	Texas Red-X phalloidin (Invitrogen, Karlsruhe, Germany), stored at -20° C
Phorbol 12-myristate 13-acetate (PMA)	2mg/ml in H_2O, stored at -20° C
ProLong Gold antifade reagent	(Invitrogen, Karlsruhe, Germany), stored at -20° C
Protein marker	High-Range Rainbow Molecular Weight Marker (GE Healthcare, München, Germany), stored at -20° C
Proteose peptone solution	10 % proteose peptone No.3 in H_2O, stored at 4° C
Red blood cell lysis buffers	0.2% or 1.6% NaCl in H_2O, stored at 4° C
Red-fluorescent latex beads with a diameter of 20 nm (20 nm LB)	FluoSpheres fluorescent microspheres, carboxylate-modified, red (580/605), 0.02 µm (Invitrogen, Karlsruhe, Germany), stored at 4° C
RPMI	1x VLE RPMI 1640 medium, stored at 4° C
Sheep blood agar plates	Stored at 4° C (Heipha, Eppelheim, Germany)
ß-mercaptoethanol (ß-ME)	ß-mercaptoethanol, min. 98%
Streptavidin	Promega (Madison, USA), stored at -20° C
TBS	10 mM Tris-HCl and 150 mM NaCl in H_2O, pH 7.4
TBS-T	0.1% Tween-20 in TBS
TRITC	Tetramethylrhodamine isothiocyanate Isomer R, 8 mM in DMSO, stored at -20° C
Trypanblue solution	1 x ready to use solution

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Trypsin-EDTA solution

10 x Trypsin-EDTA solution (Biochrom AG, Berlin), made up to 1 x using H_2O, stored at 4° C

2.1.4 Antibodies

Purified rabbit antibodies recognizing murine cation-independent M6PR, Rab7 or the E subunit of the vATPase were kindly provided by Gustav E. Lienhard (Dartmouth Medical School, Hanover, USA) (Tanner and Lienhard 1989), Marino Zerial (Max Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany) (Rink, Ghigo et al. 2005) or Dennis Brown (Harvard University, Boston, USA) (Alexander, Brown et al. 1999), respectively. Rabbit serum against p22[phox] was kindly donated by Mary Dinauer (Indiana University School of Medicine, Indianapolis, USA).

Antigen	Specification	Provider
Primary		
Cathepsin B	Purified polyclonal goat anti-mouse, WB 1:200	R&D Systems (Wiesbaden-Nordenstadt, Germany)
Cathepsin D (C-20)	Purified polyclonal goat anti-human, WB 1:200	SantaCruz Biotechnology (Heidelberg, Germany)
Cathepsin L (M-19)	Purified polyclonal goat anti-mouse, WB 1:200	SantaCruz Biotechnology (Heidelberg, Germany)
cation-independent M6PR	Purified rabbit anti-mouse, IFM 1:250, WB 1:1000	Gustav E. Lienhard
hen egg lysozyme	Purified polyclonal rabbit anti-lysozyme, WB 1:5000	Abcam (Cambridge, UK)
horseradish peroxidase	Purified polyclonal rabbit anti-HRP, WB 1:30000	Abcam (Cambridge, UK)
L. monocytogenes	Purified polyclonal rabbit anti-*L. monocytogenes*, IFM 1:250	US Biologicals (Swampscott, USA)
Lamp1	Purified monoclonal rat anti-mouse, IFM and WB 1:1000	Becton Dickinson GmbH, Heidelberg, Germany
p22[phox]	Rabbit anti-mouse serum, IFM 1:100	Mary Dinauer
Rab7	Purified rabbit anti-mouse, IFM 1:250, WB 1:200	Marino Zerial

ß-actin	Monoclonal chicken anti-mouse (clone AC-15), WB 1:10000	Sigma-Aldrich (Steinhausen, Germany)
vATPase, E subunit	Purified rabbit anti-mouse, WB 1:500	Dennis Brown

Secondary

Goat -HRP	Rabbit anti-goat IgG conjugated with HRP, WB 1:10000	Dianova (Hamburg, Germany)
Mouse -HRP	Goat anti-mouse IgG (γ chain) conjugated with HRP, WB 1:10000	Sigma-Aldrich (Steinhausen, Germany)
Rabbit -Alexa Fluor 405	Goat anti-rabbit IgG conjugated with Alexa Fluor 405, IFM 1:1000	Invitrogen (Karlsruhe, Germany)
Rabbit -Alexa Fluor 594	Chicken anti-rabbit IgG conjugated with Alexa Fluor 594, IFM 1:1000	Invitrogen (Karlsruhe, Germany)
Rabbit -HRP	Goat anti-rabbit IgG conjugated with HRP, WB 1:10000	Sigma-Aldrich (Steinhausen, Germany)
Rat -Cy3	Goat anti-rat IgG conjugated with Cy3, IFM 1:1000	Dianova (Hamburg, Germany)
Rat -HRP	Goat anti-rat IgG (H+L) conjugated with HRP, WB 1:10000	Dianova (Hamburg, Germany)

Phalloidin

Phalloidin -Texas Red-X	Phalloidin conjugated with Texas Red-X, IFM 1:1000	Invitrogen (Karlsruhe, Germany)

2.1.5 Technical equipment

Device	Specification	Provider
Blot and gel chambers	Criterion-blotter	Bio-Rad Laboratories (Hercules, USA)
Centrifuge	5417 R	Eppendorf (Hamburg, Germany)
Centrifuge	Megafuge 1.0 R	Heraeus Instruments GmbH (Düsseldorf, Germany)
Confocal laser scanning microscope	DMIRE2 with Leica Confocal Software TCS SP2 (Version 2.5.1347d)	Leica Mikrosysteme Vertrieb GmbH (Bensheim, Germany)
Developer	Curix	AGFA (Düsseldorf, Germany)
Flow cytometer	FACSCalibur with CellQuest Pro software	Becton Dickinson GmbH (Heidelberg, Germany)
Fluorescence microscope	IX81 with Analysis 3.2 software	Olympus (Hamburg, Germany)
Fluorometer	Victor^2 1420 Multilabel Counter	Wallac Oy (Turku, Finnland)
Incubator (bacteria)	Kelvitron t	Heraeus Instruments GmbH (Düsseldorf, Germany)
Incubator (cells)	Hera Cell 240	Heraeus Instruments GmbH (Düsseldorf, Germany)
Magnetic separator	OctoMACS	Miltenyi Biotec (Bergisch Gladbach, Germany)
Microscope	Axiovert 25	Carl Zeiss MicroImaging GmbH (Göttingen, Germany)
Microtiter plate reader	MRX with Revelation Software (version 4.06)	Dynex (Denkendorf, Germany)
Photometer	Genesys 20	Thermo Spectronic (Rochester, USA)
Power supply	Power Pac 3000	Bio-Rad Laboratories (Hercules, USA)

2.1.6 Kits

BCA Protein Assay kit	Thermo Spectronic (Rochester, USA)
ECL Western Blotting Detection kit	GE Healthcare (München, Germany)

2.2 Methods

All incubations were performed in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) but without antibiotics at 37° C, 5% CO_2 and water vapour saturated atmosphere unless stated otherwise.

2.2.1 Preparation of peritoneal macrophages

For *in vivo* generation of listeriocidal peritoneal macrophages (Alford, King et al. 1991), 1 ml proteose peptone solution was injected into the peritoneal cavity of mice two days before sacrifice by cervical dislocation. Proteose peptone-elicited peritoneal exudate cells were harvested by peritoneal lavage with ice cold PBS. After red blood cell lysis in 5 ml 0.2% NaCl in H_2O for 30 seconds, isotonic conditions were reconstituted with 5 ml 1.6% NaCl in H_2O. Viable peritoneal macrophages were counted using Trypanblue exclusion in a Neubauer chamber.

2.2.2 Immunomagnetic enrichment of peritoneal macrophages

Proteose peptone-elicited peritoneal macrophages were enriched from peritoneal exudate cells by magnetic cell sorting using CD11b-specific monoclonal antibodies conjugated to paramagnetic beads (CD11b MicroBeads) according to the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). After elution from the MS column, peritoneal macrophages were counted using Trypanblue exclusion in a Neubauer chamber.

2.2.3 Preparation of bone marrow-derived macrophages

Bone marrow cells prepared from the femurs of mice were incubated in RPMI supplemented with 10% FCS, 1% penicillin/streptomycin, 1% HEPES and 15% L929-conditioned medium for 10 days to differentiate into bone marrow-derived macrophages (BMDM). Antibiotics were removed 24 h prior to infection of BMDM.

2.2.4 Cell culture

Simian virus (SV) 40-transformed C57BL/6 ASMase$^{-/-}$ and wt mouse embryonic fibroblasts (MEF) were grown and maintained in tissue culture flasks and passaged weekly after treatment with trypsin-EDTA.

2.2.5 Labelling of *L. monocytogenes* with fluorescent dyes

L. monocytogenes were grown overnight in BHI medium, resuspended in fresh BHI medium and harvested during mid-log phase. After washing once with PBS, density of *L. monocytogenes* was estimated by OD measurement at 600 nm (OD_{600} 1 = 5 x 10^8 CFU/ml). *L. monocytogenes* at a density of 1 x 10^9 CFU/ml were incubated with either 2 mg/ml of FITC isomer I or 1 mM TRITC isomer R and 1 mM Oregon Green 488-X succinimidyl ester in 0,1 M $NaHCO_3$ in H_2O, pH 9 for 1 h at room temperature (RT). Unbound dye was removed by repeated washing with PBS. Stocks of labelled *L. monocytogenes* were stored in PBS supplemented with 10% DMSO at -80° C until further use. For each experiment, a fresh aliquot was thawed.

2.2.6 Preparation of heat-killed *L. monocytogenes*

Heat-killed *L. monocytogenes* (HKLM) were prepared by incubating *L. monocytogenes* at 60° C for 60 min. Inactivation of HKLM was proven by plating on sheep blood agar plates before use.

2.2.7 Immunofluorescence microscopy (IFM)

Proteose peptone-elicited peritoneal macrophages were allowed to adhere to sterile 15x15 mm cover slips at a density of 2 x 10^5 cells/well in 12-well plates. Non-adherent cells were removed after 1 h by washing once with PBS. Afterwards, either viable or heat-killed *L. monocytogenes* were added at a MOI of 5 in ice cold DMEM with 10% FCS and 5% normal mouse serum (NMS). Adherence of *L. monocytogenes* to macrophages was synchronized by centrifugation at 850 g, 4° C for 5 min. Subsequently, non-adherent *L. monocytogenes* were removed by triple washing with ice cold PBS. Infected macrophages were incubated in pre-warmed DMEM with 10% FCS. At distinct times after infection, samples were fixed in 3% PFA in PBS for 20 min at RT. Cells were permeabilized with 0.1% saponin in PBS for 20 min, blocked with blocking buffer (3% BSA and 0.1% saponin in PBS) for 15 min and then stained with the primary antibody diluted in blocking buffer for 30 min at RT. After

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triple washing with 0.1% saponin in PBS, the secondary antibody diluted in blocking buffer was added for 30 min at RT. Samples were washed three times with 0.1% saponin in PBS and then mounted on glass microscopic slides in ProLong Gold antifade reagent. Sections of the preparations were analysed by confocal laser scanning microscopy.

2.2.8 Pulse/chase labelling of lysosomes with fluid phase markers

Peritoneal macrophages at a density of 2×10^5 cells/ml were pulsed for 1 h with either 0.6 mg/ml 3 kDa dextran conjugated to TexasRed, 30 µg/ml chicken ovalbumin conjugated to TexasRed or 1 mg/ml 70 kDa dextran conjugated to TexasRed in DMEM with 10% FCS or with 5×10^{10} red-fluorescent latex beads with a diameter of 20 nm in DMEM without FCS. Non-endocytosed fluid phase markers were removed by triple washing with PBS. The fluorescent fluid phase markers were chased into lysosomes by incubation in fresh medium for 2 h. Afterwards, the cells were prepared for immunofluorescence microscopy as described above.

To quantify uptake of fluid phase markers, immunomagnetically enriched peritoneal macrophages were pulse/chase-labelled as described above and then lysed in lysis buffer for 30 min on ice. Fluorometric analysis of macrophage lysates was performed in a Wallac Victor^2 1420 Multilabel Counter.

Stoke's radii as an indicator for the diameter of dextrans and ovalbumin were calculated as described by Venturoli and Rippe (Venturoli and Rippe 2005).

2.2.9 Western blotting (WB)

Protein concentration in samples was determined by BCA Protein Assay according to the instructions of the supplier (Thermo Scientific, Rockford, USA). Equal amounts of protein in 1x Laemmli buffer were denatured for 5 min at 95° C. Samples were loaded onto NuPAGE Novex 10% Bis-Tris Midi gels and run under standard conditions. For immunoblotting, proteins were electrophoretically transferred to an Immobilon-P^{SQ} transfer membrane at 250 mA for 1.5 h. Membranes were blocked with blocking buffer for 1h at RT before incubation with primary antibody in blocking buffer overnight at 4° C. After triple washing with TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in blocking buffer for 1h at 4° C. Unbound antibodies were removed by washing three times with TBS-T

and twice with TBS. The immune complex was visualised by an enhanced chemiluminescence system (ECL Western Blotting reagent) and detected by Amersham ECL hyperfilm. A commercial protein marker was used for identification of protein size.

Specific bands on immunoblots were quantified by densidometry with imageJ (NIH, USA).

2.2.10 Preparation of phagosomes containing *L. monocytogenes*

$1x10^8$ MEF or BMDM were infected with the $\Delta prfA$-mutant of *L. monocytogenes* ($\Delta prfA$-*L. monocytogenes*) at a MOI of 10 for 1 h or 20 min, respectively. After removal of extracellular *L. monocytogenes* by triple washing with PBS, cells were incubated in fresh medium for 2 h or 100 min, respectively. Phagosomes containing $\Delta prfA$-*L. monocytogenes* were prepared as described by Lührmann *et al.* (Luhrmann and Haas 2000) and analysed via Western blotting.

2.2.11 Measurement of pH in phagosomes containing *L. monocytogenes*

Immunomagnetically enriched, proteose peptone-elicited macrophages were co-incubated at a MOI-equivalent of 50 with HKLM double-labelled with Oregon Green 488 and TRITC in DMEM with 10% FCS and 5% NMS for 20 min. Non-phagocytosed HKLM were removed by washing with ice cold PBS. At distinct times after co-incubation, the ratio of pH-dependent Oregon Green 488 fluorescence intensity to pH-independent TRITC fluorescence intensity in MES buffer was determined by flow cytometry. A calibration curve of samples treated with 50 µM monensin and 10 µM nigericin for 15 min in MES buffer of graded pH (3.0 to 7.5) was used to calculate the pH in phagosomes containing HKLM (Vergne, Constant et al. 1998; Holopainen, Saarikoski et al. 2001).

2.2.12 Measurement of proteolytic activity

Proteose peptone-elicited peritoneal macrophages at a density of $1,5x10^6$ cells/ml were co-incubated with 0.5 mg/ml hen egg lysozyme or horseradish peroxidase in DMEM with 5% FCS. After 20 min, non-endocytosed protein was removed by triple washing with ice cold PBS. After distinct times of incubation in DMEM with 5% FCS,

macrophages were washed twice with PBS, lysed in Laemmli buffer for 5 min and subsequently analysed by Western blotting.

To determine intraphagosomal proteolytic activity, carboxylated 1 μm silica beads were coated with human IgG and streptavidin and labelled with Alexa Fluor 594-succinimidyl ester and the peptide substrate (Biotin-LC-Phe-Arg)$_2$-rhodamine 110 as described by Yates *et al.* (Yates, Hermetter et al. 2005). Immunomagnetically enriched, proteose peptone-elicited macrophages at a density of $0,75 \times 10^8$ cells/ml were co-incubated with labelled beads for 3 min at 37° C in a very small volume to facilitate bead phagocytosis. The concentration of beads was titrated to achieve an average of 1-2 beads per macrophage and quantitative bead uptake was assessed via fluorescence microscopy. Rhodamine 110 and Alexa Fluor 594 fluorescence intensity was determined in a Wallac Victor^2 1420 Multilabel Counter immediately (t_0) and at specific time points (t) of co-incubation with beads. Proteolysis of the substrate was expressed as ratio of specific rhodamine 110 fluorescence intensity t / t_0 and Alexa Fluor 594 fluorescence intensity.

2.2.13 Quantification of reactive oxygen intermediates

Superoxide production of resident or IFN-γ pre-activated, immunomagnetically enriched peritoneal macrophages after stimulation with PMA was measured by the reduction of ferricytochrome c as described by *Kruisbeek et al.* (Kruisbeek and Vogel 1999).

2.2.14 Quantification of reactive nitrogen intermediates

Production of nitrite by resident or IFN-γ pre-activated, immunomagnetically enriched peritoneal macrophages after stimulation with HKLM at a MOI-equivalent of 10 was determined using the Greiss reagent as described by *Kruisbeek et al.* (Kruisbeek and Vogel 1999).

2.2.15 Statistical analysis

For statistical analysis, the data were subjected to one-way analysis of variance (ANOVA) using SigmaStat 3.5 (Systat Software GmbH, Erkrath, Germany).

3 Results

3.1 Impaired inactivation of *Listeria monocytogenes* despite normal generation of ROI and RNI in ASMase$^{-/-}$ macrophages

To elucidate the role of ASMase in the listeriocidal activity of macrophages, we investigated both the oxidative and non-oxidative bactericidal effector mechanisms of proteose peptone-elicited peritoneal macrophages from ASMase$^{-/-}$ and wild type (wt) mice.

Deficiency in ASMase impairs mannose 6-phosphate receptor-mediated endocytosis in macrophages (Dhami and Schuchman 2004) and internalization of *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* into human epithelial cells (Grassme, Gulbins et al. 1997; Grassme, Jendrossek et al. 2003). Thus, we first examined phagocytic uptake of *L. monocytogenes* into macrophages from ASMase$^{-/-}$ and wt mice.

ASMase$^{-/-}$ and wt macrophages phagocytosed fluorescein isothiocyanate (FITC)-labelled *L. monocytogenes* with identical kinetics (Fig. 2A) and in similar numbers (Fig. 2B, t = 0 min). Importantly, while wt macrophages restricted intracellular proliferation, ASMase$^{-/-}$ macrophages allowed significant multiplication of *L. monocytogenes* (Fig. 2B). Correspondingly, *L. monocytogenes* colocalized with actin in ASMase$^{-/-}$ macrophages but not in wt macrophages which indicates escape of *L. monocytogenes* from phagosomes into the cytosol of ASMase$^{-/-}$ macrophages (Fig. 2C and D). Together, these data show that ASMase$^{-/-}$ macrophages efficiently phagocytose *L. monocytogenes* but subsequently fail to inactivate the bacteria.
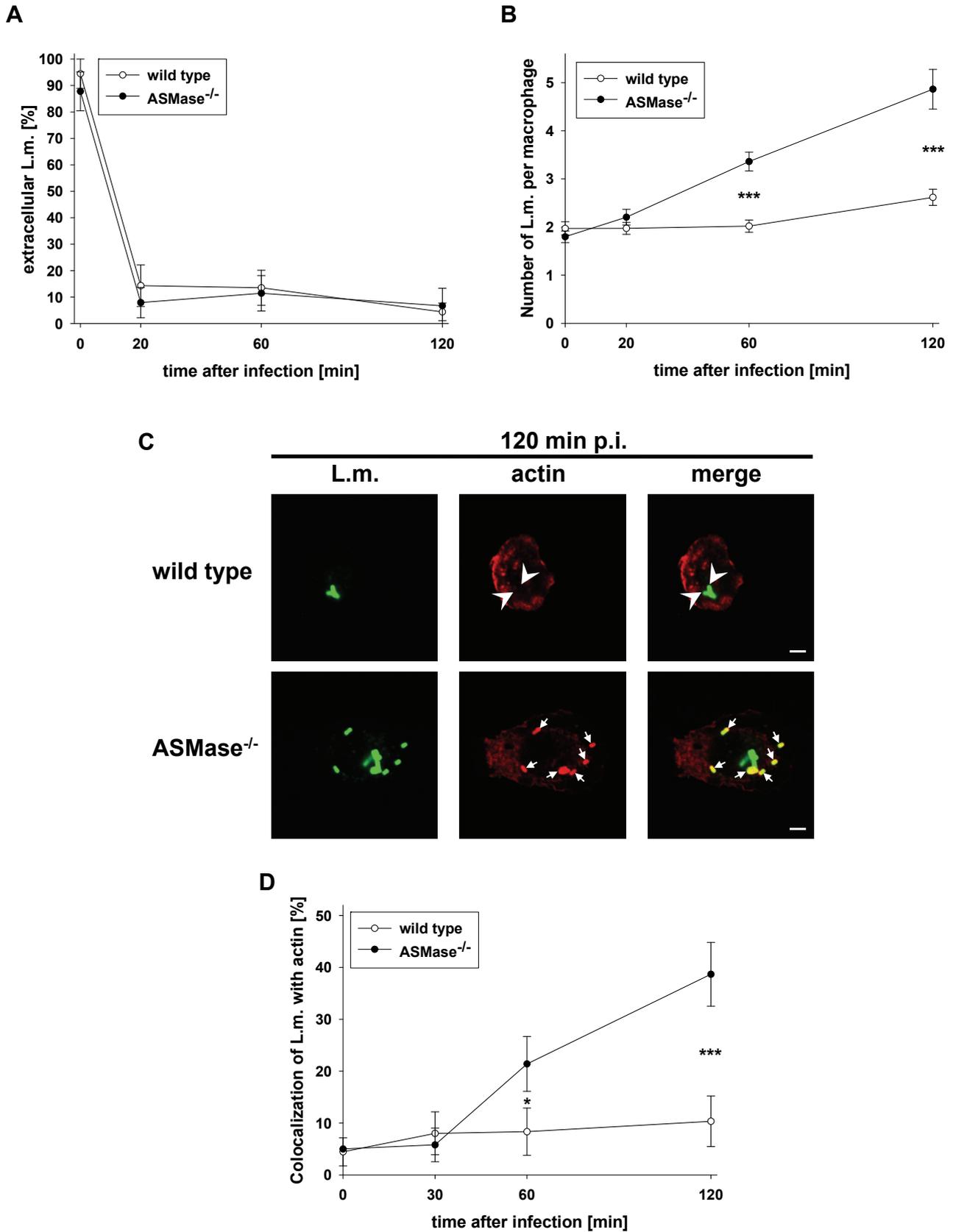
A

B

C

D

Fig. 2: *Listeria monocytogenes* escapes from phagosomes, recruits actin and rapidly proliferates in ASMase^{-/-} but not wt macrophages.

(Legend of Fig. 2 / continued)

Proteose peptone-elicited peritoneal macrophages of ASMase$^{-/-}$ or wt littermates were infected at a MOI of 5 with FITC-labelled *L. monocytogenes* (L.m.). At the indicated times after infection, samples were fixed and analysed by confocal laser scanning microscopy. (A) Kinetics of the decrease in extracellular *L. monocytogenes*. Extracellular *L. monocytogenes* were stained with an antibody against *L. monocytogenes* without permeabilization. *L. monocytogenes* double-stained with antibody and FITC were considered extracellular, FITC-only *L. monocytogenes* intracellular. (B) Kinetics of the number of intracellular *L. monocytogenes* per macrophage. (C) Representative micrographs indicating colocalization (arrows) or non-colocalization (arrow heads) of *L. monocytogenes* with phalloidin-stained actin at 120 min after infection. Bars are 4 μm. (D) Kinetics of the colocalization of *L. monocytogenes* with phalloidin-stained actin. At each time point, a minimum of 100 cells from three independent experiments was analysed. Data are shown as mean +/- s.e. Statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA. P-values are indicated by * ($p < 0.05$) or *** ($p < 0.001$).

ROI are generated after assembly of the NADPH oxidase complex in the phagosomal membrane. *L. monocytogenes* colocalized with the catalytic p22^{phox} subunit of the NADPH oxidase complex with similar kinetics in ASMase$^{-/-}$ and wt macrophages (Fig. 3A and B) indicating unimpaired recruitment of p22^{phox} to the phagosomal membrane. Furthermore, both resident and IFN-γ pre-activated ASMase$^{-/-}$ macrophages generated similar amounts of superoxide (O_2^-) as wt macrophages after stimulation with PMA (Fig. 3C) showing unimpaired generation of ROI. Additionally, ASMase$^{-/-}$ and wt macrophages generated similar amounts of NO after pre-activation with IFN-γ and stimulation with HKLM (Fig. 3D) indicating unimpaired generation of RNI. Thus, ASMase$^{-/-}$ macrophages generate normal amounts of oxidative bactericidal effector molecules but cannot kill *L. monocytogenes* suggesting that non-oxidative bactericidal effector mechanisms might depend on ASMase activity.
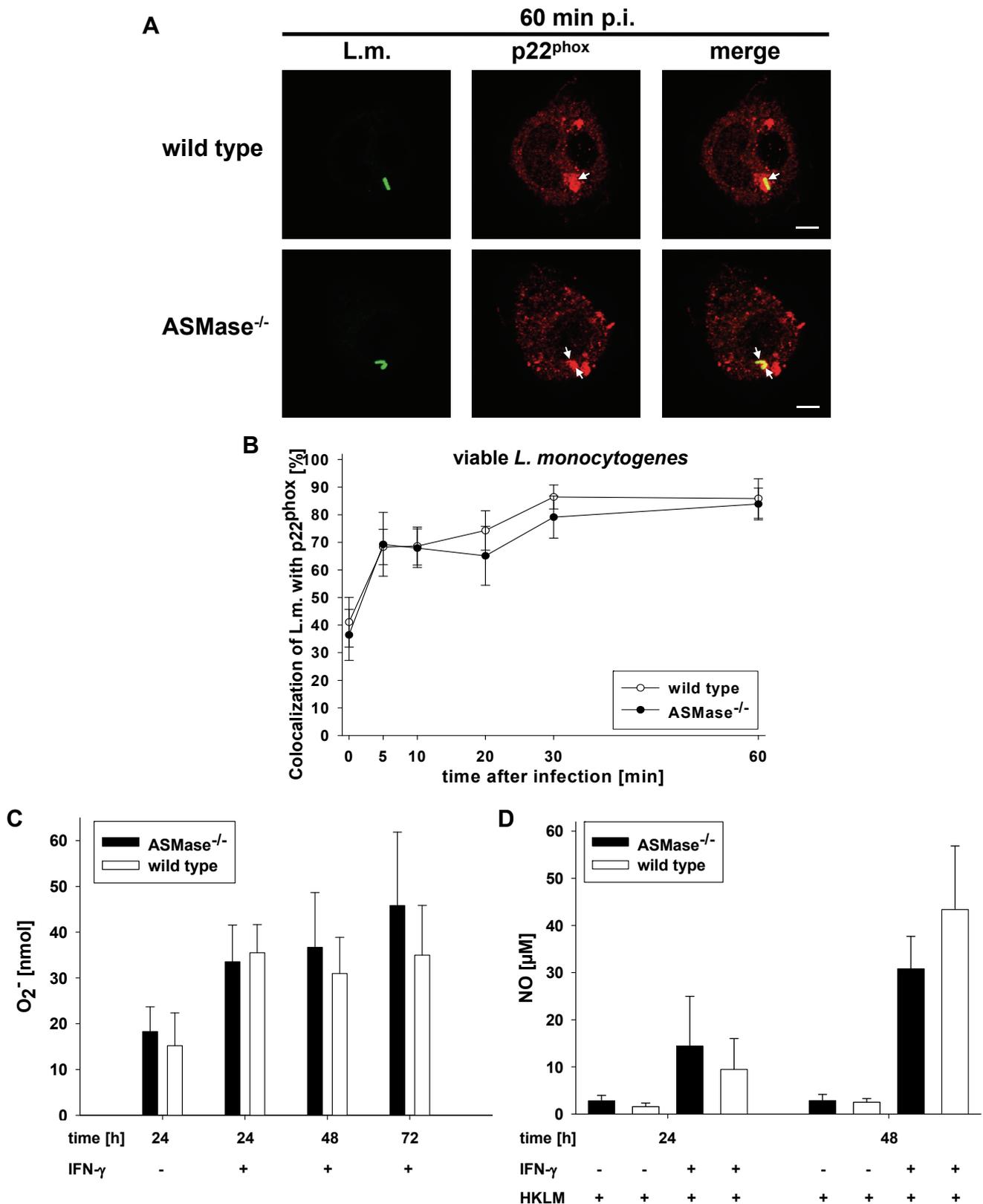
A

60 min p.i.

L.m.   p22phox   merge

wild type

ASMase^{-/-}

B

viable *L. monocytogenes*

C

D

Fig. 3: Normal activity of oxidative effector mechanisms in ASMase^{-/-} macrophages.

(A, B) Proteose peptone-elicited peritoneal macrophages from ASMase^{-/-} or wt littermates were infected at a MOI of 5 with FITC-labelled *L. monocytogenes*. At the

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indicated times after infection, samples were fixed and analysed by confocal laser scanning microscopy. (A) Representative micrographs indicating colocalization (arrows) of *L. monocytogenes* with p22[phox] at 60 min after infection. Bars are 4 µm. (B) Kinetics of the colocalization of *L. monocytogenes* with p22[phox]. At each time point, a minimum of 100 phagosomes from three independent experiments was analysed. Percentage of *L. monocytogenes* per macrophage colocalizing with p22[phox] are shown as mean +/- s.e. (C) Generation of superoxide by immunomagnetically enriched, resident or IFN-γ pre-activated ASMase^{-/-} and wt macrophages after stimulus with PMA was measured after 24, 48 and 72 h by the reduction of ferricytochrome c. Results from four independent experiments are shown as mean +/- s.e. (D) Generation of NO by immunomagnetically enriched, resident or IFN-γ pre-activated ASMase^{-/-} and wt macrophages stimulated with HKLM was measured after 24 and 48 h using the Greiss reagent. Results from three independent experiments are shown as mean +/- s.e.

3.2 Impaired maturation of phagosomes containing *L. monocytogenes* into phagolysosomes in ASMase^{-/-} macrophages

Non-oxidative bactericidal effector mechanisms exerted by acid hydrolases, particularly the lysosomal protease cathepsin D, are essential for listeriocity of macrophages (del Cerro-Vadillo, Madrazo-Toca et al. 2006). These acid hydrolases are transferred from lysosomes into phagosomes at the end of the phagosomal maturation process (Haas 2007).

To investigate maturation of phagosomes into phagolysosomes, we analysed the kinetics of the colocalization of *L. monocytogenes* with the (phago)lysosomal marker 'lysosome-associated membrane protein 1' (Lamp1) in ASMase^{-/-} and wt macrophages. In wt macrophages, more than 90 % of the phagocytosed *L. monocytogenes* colocalized with Lamp1 from 30 min post infection (p.i.) on (Fig. 4A and B). In contrast, only 50% of the phagocytosed *L. monocytogenes* colocalized with Lamp1 in ASMase^{-/-} macrophages indicating impaired maturation of phagosomes into phagolysosomes.

The reduced colocalization of *L. monocytogenes* with Lamp1 in ASMase^{-/-} macrophages could be due to (i) interference of *L. monocytogenes* with the phagosomal maturation (Alvarez-Dominguez, Barbieri et al. 1996; Alvarez-Dominguez, Roberts et al. 1997; Prada-Delgado, Carrasco-Marin et al. 2005; Alvarez-Dominguez, Madrazo-Toca et al. 2008) (ii) escape of *L. monocytogenes* from

phagosomes (Tilney and Portnoy 1989) or (iii) a cellular defect in the maturation of phagosomes into phagolysosomes.

To discriminate between these alternatives, we performed two additional series of experiments. First, we analysed the colocalization of heat-killed *L. monocytogenes* (HKLM) with Lamp1 because HKLM can neither actively interfere with the phagosomal maturation nor escape from phagosomes (Alvarez-Dominguez, Roberts et al. 1997). As illustrated in Fig. 4C, HKLM colocalized significantly less frequently with Lamp1 in ASMase$^{-/-}$ than in wt macrophages at 30 min and 60 min after co-incubation of HKLM with macrophages. Afterwards, the percentage of HKLM colocalizing with Lamp1 in ASMase$^{-/-}$ macrophages slowly increased to reach almost the level observed in wt macrophages at 120 min after co-incubation. Second, we studied colocalization of the viable but apathogenic listeriolysin O-deficient Δ*hly*-strain of *L. monocytogenes* (Δ*hly-L. monocytogenes*) with Lamp1. Listeriolysin O (LLO) is the major virulence factor required for escape of *L. monocytogenes* from phagosomes (Leimeister-Wachter, Haffner et al. 1990; Beauregard, Lee et al. 1997). Fig. 4D shows that Δ*hly-L. monocytogenes* colocalized significantly less frequently with Lamp1 in ASMase$^{-/-}$ than in wt macrophages from 20 min p.i. on. These data show that reduced colocalization of *L. monocytogenes* with Lamp1 in ASMase$^{-/-}$ macrophages is independent of the viability or the virulence of *L. monocytogenes*.
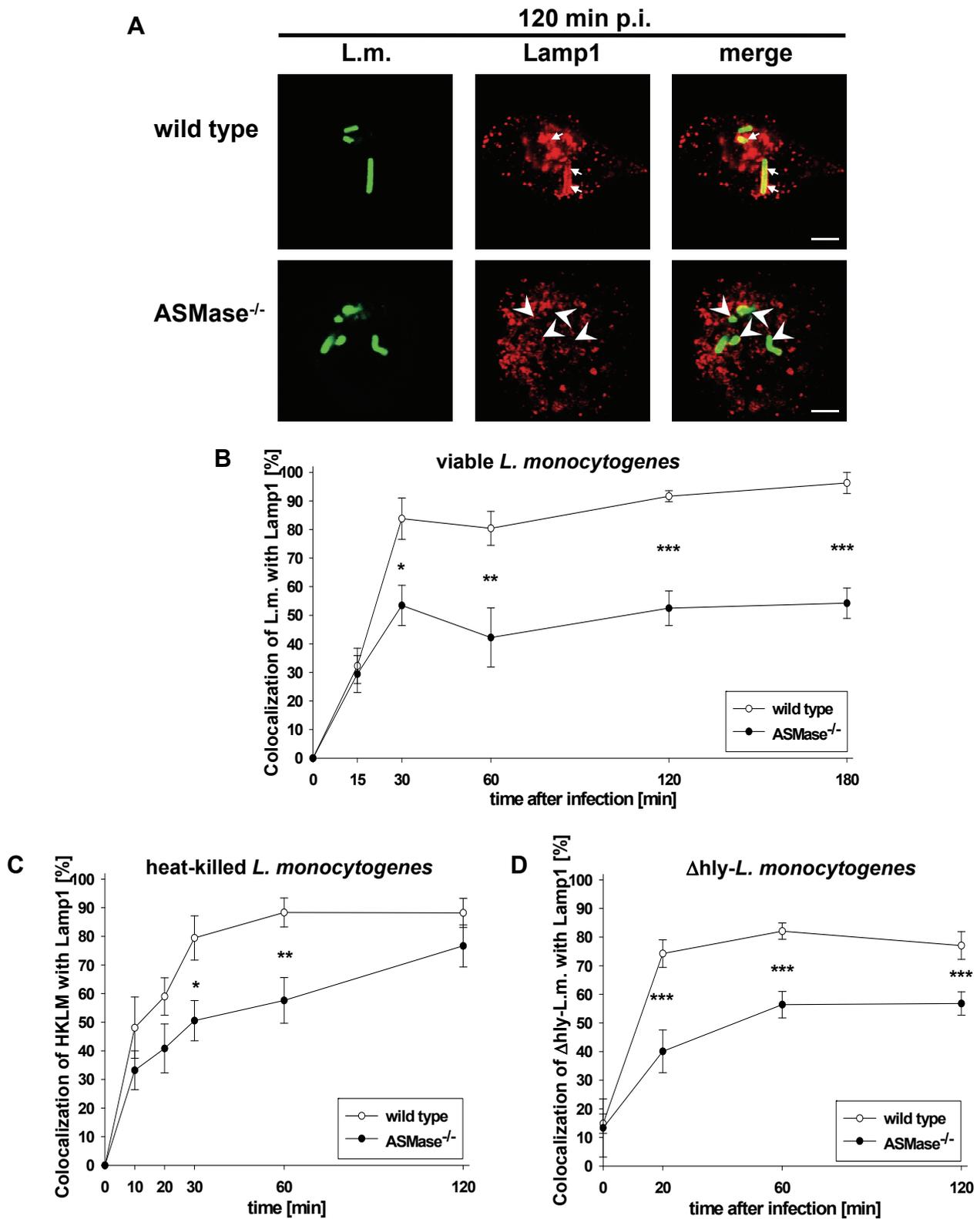
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Fig. 4: Reduced colocalization of viable, heat-killed and *Δhly-L. monocytogenes* with Lamp1 in ASMase^{-/-} macrophages.

Proteose peptone-elicited peritoneal macrophages from ASMase$^{-/-}$ or wt littermates were infected at a MOI of 5 with FITC-labelled (A, B) wild type or

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(D) Δ*hly-L. monocytogenes* or (C) incubated with an equivalent number of heat-killed *L. monocytogenes* (HKLM). At the indicated times after infection, samples were fixed and analysed by confocal laser scanning microscopy. (A) Representative micrographs indicating colocalization (arrows) or non-colocalization (arrow heads) of viable wild type *L. monocytogenes* with Lamp1 at 120 min after infection. Bars are 4 μm. (B, C, D) Kinetics of the colocalization of (B) viable wt *L. monocytogenes*, (C) HKLM or (D) Δ*hly-L. monocytogenes* with Lamp1. At each time point, a minimum of 100 phagosomes from three independent experiments was analysed. Percentage of Listeria per macrophage colocalizing with Lamp1 are shown as mean +/- s.e. The statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA. P-values are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

To further investigate whether a cellular defect of ASMase$^{-/-}$ macrophages impairs the maturation of phagosomes containing *L. monocytogenes* into phagolysosomes, we analysed acquisition of the 'cation-independent mannose 6-phosphate receptor' (M6PR) by phagosomes containing *L. monocytogenes*. M6PR is present on late phagosomes but not on phagolysosomes (Haas 2007).

In both ASMase$^{-/-}$ and wt macrophages, more than 60% of the phagocytosed *L. monocytogenes* colocalized with M6PR within 20 min p.i. (Fig. 5A and B). Afterwards, most phagosomes containing *L. monocytogenes* in wt macrophages lost colocalization with M6PR within just 10 min (30% at 30 min p.i.). In sharp contrast, phagosomes containing *L. monocytogenes* in ASMase$^{-/-}$ macrophages remained positive for M6PR until at least 60 min p.i. and slowly lost colocalization with M6PR between 60 min and 120 min post infection.

To discriminate whether this late loss of colocalization of *L. monocytogenes* with M6PR in ASMase$^{-/-}$ macrophages was caused by escape of *L. monocytogenes* from phagosomes or by maturation of late phagosomes into phagolysosomes, we used HKLM and Δ*hly-L. monocytogenes*. In both ASMase$^{-/-}$ and wt macrophages, more than 50% of HKLM colocalized with M6PR within 20 min after co-incubation (Fig. 5C). Afterwards, the percentage of HKLM colocalizing with M6PR in wt macrophages dropped significantly to about 30% at 60 min after co-incubation and 15% at 120 min after co-incubation. In ASMase$^{-/-}$ macrophages, however, phagosomes containing HKLM remained positive for M6PR until at least 120 min after co-incubation. Similarly, after infection with Δ*hly-L. monocytogenes*, colocalization with M6PR was sustained at peak levels in ASMase$^{-/-}$ macrophages but rapidly declined in wt

macrophages (Fig. 5D). These data show that colocalization of viable *L. monocytogenes* with M6PR in ASMase$^{-/-}$ macrophages beyond 60 min p.i. dropped only because the bacteria escaped from phagosomes. Both HKLM and Δhly-*L. monocytogenes* remained within M6PR-positive phagosomes in ASMase$^{-/-}$ macrophages further indicating an impaired maturation of phagosomes into phagolysosomes.
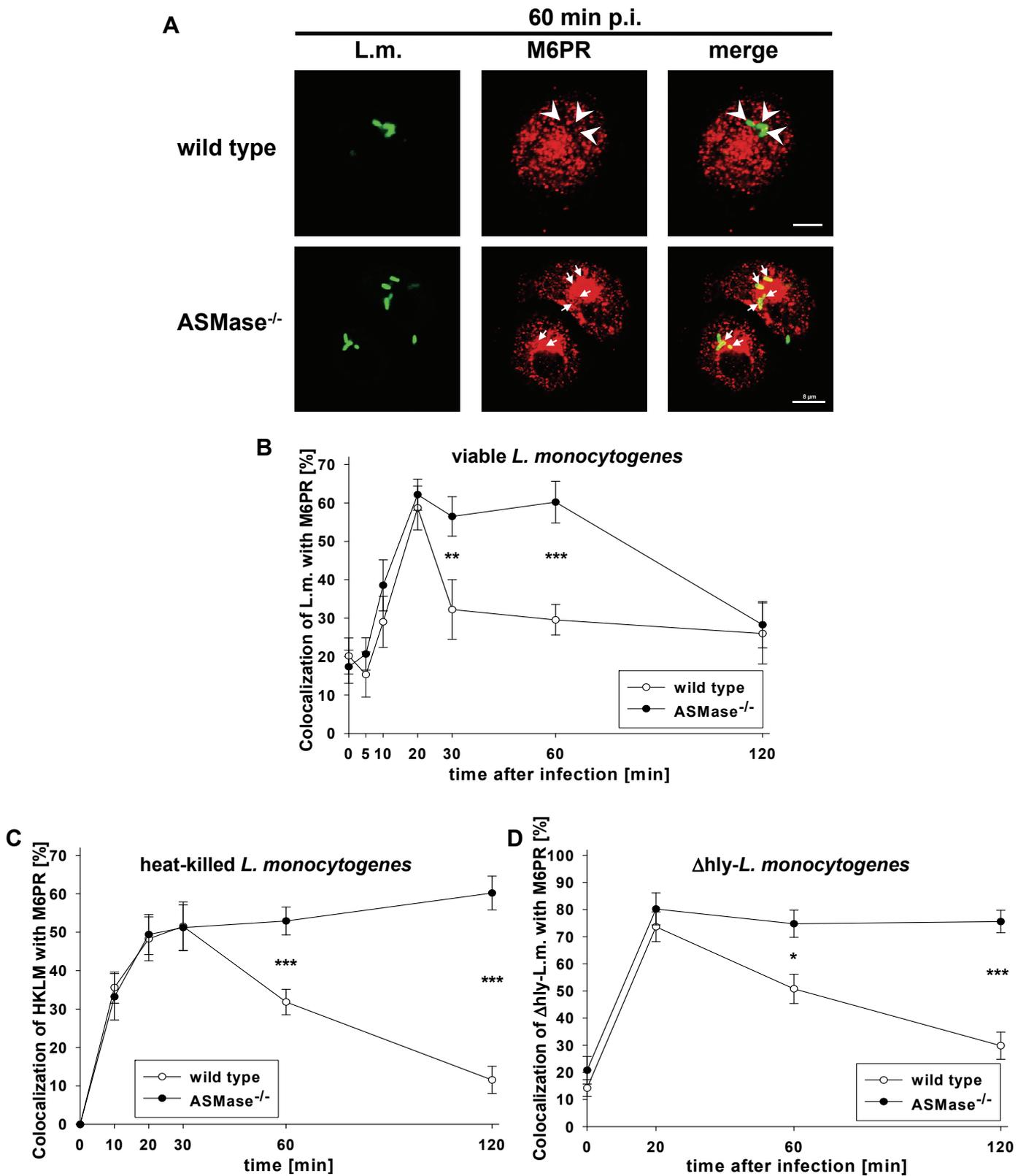
A

60 min p.i.

L.m.   M6PR   merge

wild type

ASMase$^{-/-}$

B viable *L. monocytogenes*

C heat-killed *L. monocytogenes*

D Δhly-*L. monocytogenes*

Fig. 5: Prolonged colocalization of viable, heat-killed and Δhly-L. monocytogenes with M6PR in ASMase$^{-/-}$ macrophages.

Proteose peptone-elicited peritoneal macrophages from ASMase$^{-/-}$ or wt littermates

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were infected at a MOI of 5 with FITC-labelled (A, B) wild type or (D) Δ*hly-L. monocytogenes* or (C) incubated with an equivalent number of heat-killed *L. monocytogenes* (HKLM). At the indicated times p.i., samples were fixed and analysed by confocal laser scanning microscopy. (A) Representative micrographs indicating colocalization (arrows) or non-colocalization (arrow heads) of viable wt *L. monocytogenes* with M6PR at 60 min after infection. Bars are 4 μm. (B, C, D) Kinetics of the colocalization of (B) viable wt *L. monocytogenes*, (C) HKLM or (D) Δ*hly-L. monocytogenes* with M6PR. At each time point, a minimum of 100 phagosomes from three independent experiments was analysed. Percentage of Listeria per macrophage colocalizing with M6PR are shown as mean +/- s.e. The statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA. P-values are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

The small GTPase Rab7 mediates the maturation of late phagosomes into phagolysosomes (Mullock, Bright et al. 1998; Bucci, Thomsen et al. 2000; Ceresa and Bahr 2006). To investigate the role of Rab7 in the phagosomal maturation defect of ASMase$^{-/-}$ macrophages, we analysed the colocalization of *L. monocytogenes* with Rab7. In both ASMase$^{-/-}$ and wt macrophages, more than 70% of viable *L. monocytogenes* (Fig. 6A and B), HKLM (Fig. 6C) or Δ*hly-L. monocytogenes* (Fig. 6D) colocalized with Rab7 at 20 min post infection. Afterwards, phagosomes containing *L. monocytogenes* lost Rab7 already at 30 min p.i. in wt macrophages but remained positive for Rab7 until 120 min p.i. in ASMase$^{-/-}$ macrophages. These data confirm a block in the maturation of phagosomes at the late phagosomal stage in ASMase$^{-/-}$ macrophages.
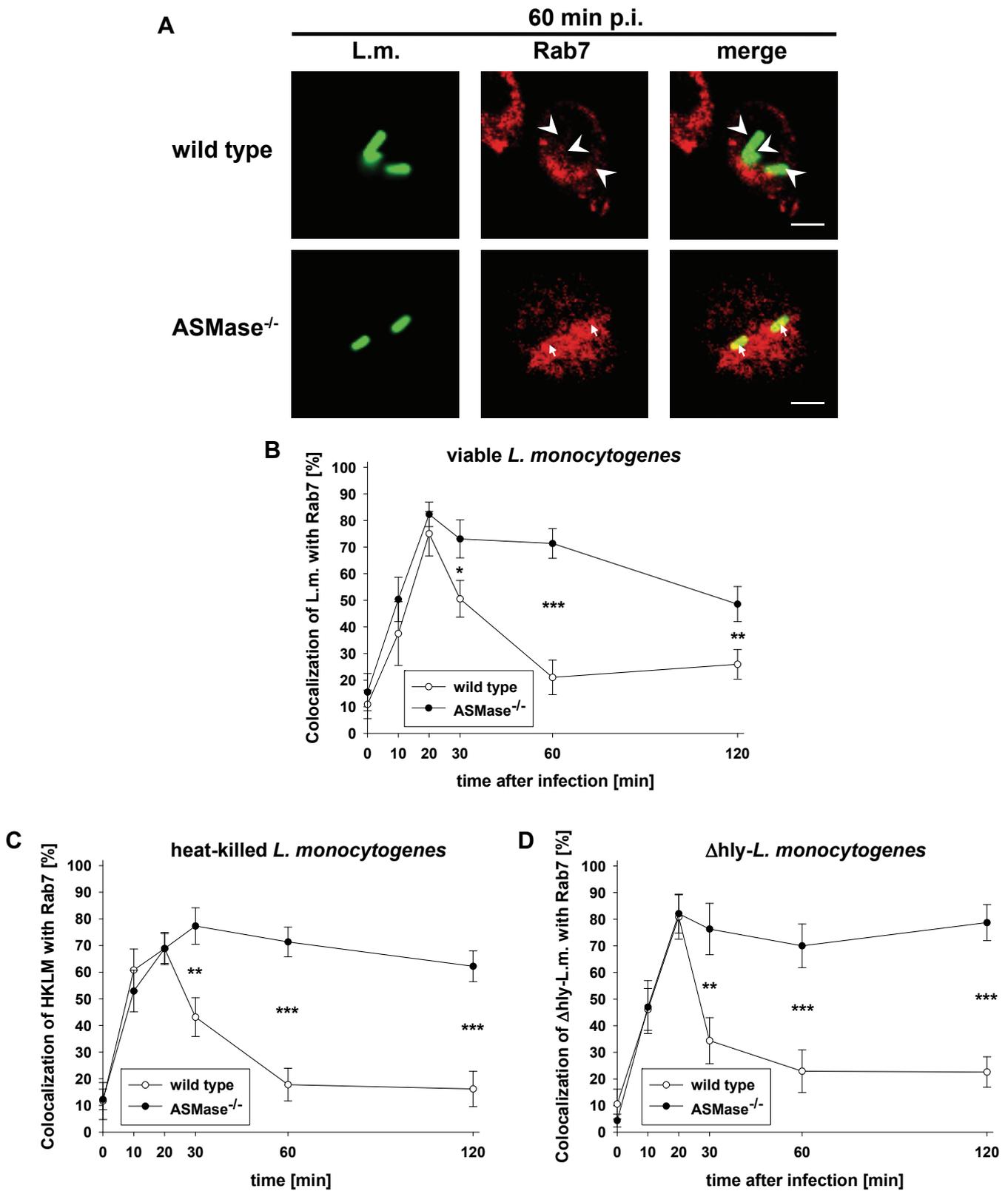
A 60 min p.i.

B viable *L. monocytogenes*

C heat-killed *L. monocytogenes*

D Δhly-*L. monocytogenes*

Fig. 6: Sustained colocalization of viable, heat-killed and *Δhly-L. monocytogenes* with Rab7 in ASMase^{-/-} macrophages.

Proteose peptone-elicited peritoneal macrophages from ASMase^{-/-} or wt littermates

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were infected at a MOI of 5 with FITC-labelled (A, B) wild type or (D) Δ*hly-L. monocytogenes* or (C) incubated with an equivalent number of heat-killed *L. monocytogenes* (HKLM). At the indicated times p.i., samples were fixed and analysed by confocal laser scanning microscopy. (A) Representative micrographs indicating colocalization (arrows) or non-colocalization (arrow heads) of viable *L. monocytogenes* with Rab7 at 60 min after infection. Bars are 4 μm. (B, C, D) Kinetics of the colocalization of (B) viable wt *L. monocytogenes*, (C) HKLM or (D) Δ*hly-L. monocytogenes* with Rab7. At each time point, a minimum of 100 phagosomes from three independent experiments was analysed. Percentage of Listeria per macrophage colocalizing with Rab7 are shown as mean +/- s.e. The statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA. P-values are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Acidification indicates functional phagosomal maturation independent of stage-specific marker proteins. After generation of phagosomes, the phagosomal lumen acidifies to a pH between 6.5 and 6.0 in early phagosomes, between 6.0 and 5.0 in late phagosomes and finally 5.0 to 4.0 in phagolysosomes (Haas 2007). To determine the pH of phagosomes containing *L. monocytogenes* in ASMase$^{-/-}$ and wt macrophages, we analysed the H$^+$-mediated quenching of the fluorescence of Oregon Green 488-labelled HKLM by flow cytometry. In wt macrophages, the pH in phagosomes containing HKLM dropped from about 5.5 at 20 min after co-incubation of HKLM with macrophages to about 4.0 at 120 min after co-incubation (Fig. 7). In ASMase$^{-/-}$ macrophages, however, phagosomes containing HKLM did not acidify below a pH of about 5.0.
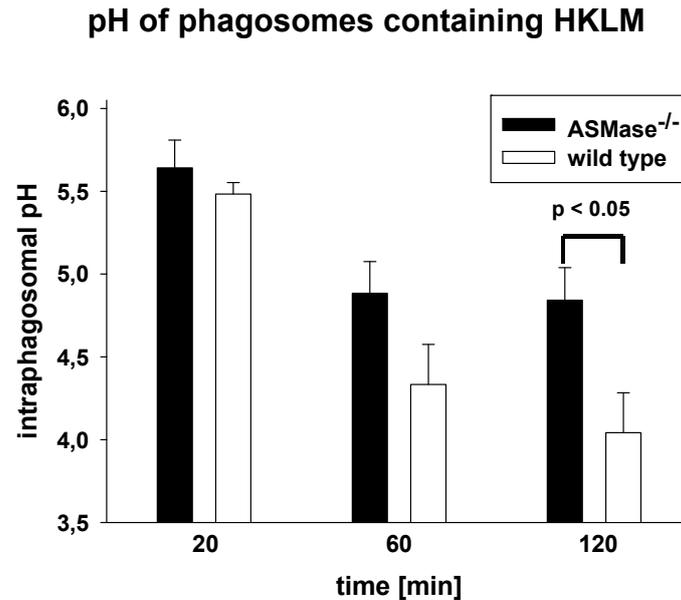
pH of phagosomes containing HKLM

Fig. 7: Impaired acidification of phagosomes containing HKLM in ASMase$^{-/-}$ macrophages.

Immunomagnetically enriched, proteose peptone-elicited peritoneal macrophages were co-incubated at a MOI-equivalent of 50 with HKLM double-labelled with Oregon Green 488 and TRITC. At the indicated times after co-incubation, fluorescence intensities of Oregon Green 488 and TRITC were determined by flow cytometry. Intraphagosomal pH was calculated as described in the Methods section (2.2.11) by comparing H$^+$-mediated quenching of Oregon Green 488 fluorescence to a standard curve. Results from four independent experiments are shown as mean +/- s.e. The statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA.

Together, our data show that phagosomes containing *L. monocytogenes* fail to mature into phagolysosomes in ASMase$^{-/-}$ macrophages as assessed by acquisition and loss of marker molecules specific for distinct stages of phagosomal maturation as well as acidification.

3.3 Impaired transfer of lysosomal cargo into phagosomes containing *L. monocytogenes* in ASMase$^{-/-}$ macrophages

Fusion of late phagosomes with lysosomes leads to the transfer of intraluminal cargo molecules from lysosomes into phagosomes (Vieira, Botelho et al. 2002; Scott, Botelho et al. 2003; Haas 2007). Particularly, the transfer of lysosomal acid hydrolases is crucial for the generation of bactericidal phagolysosomes (Reeves, Lu et al. 2002; Prada-Delgado, Carrasco-Marin et al. 2005; Segal 2005; del Cerro-Vadillo, Madrazo-Toca et al. 2006).

To functionally characterize the role of ASMase in the fusion of phagosomes with lysosomes, we pre-loaded lysosomes of both ASMase$^{-/-}$ and wt macrophages with fluorescent fluid phase markers and investigated their transfer into phagosomes containing *L. monocytogenes*. Colocalization of the fluid phase markers with Lamp1 confirmed lysosomal localization and quantitative fluorometry showed equal loading of lysosomes with fluid phase markers in ASMase$^{-/-}$ and wt macrophages (Fig. 8A).

First, we investigated the transfer of a low molecular weight marker, dextran of 3.000 Da MW conjugated to TexasRed (3 kDa TRD; about 3 nm in diameter), from lysosomes into phagosomes containing HKLM. The percentage of HKLM colocalizing with 3 kDa TRD was significantly lower in ASMase$^{-/-}$ macrophages than in wt macrophages at 30 min after co-incubation and 120 min after co-incubation (Fig. 8B).

Second, as the exchange of fluid phase markers between vesicular compartments may depend on the size of the marker molecule (Wang and Goren 1987; Desjardins, Nzala et al. 1997; Bright, Gratian et al. 2005), we studied the transfer of latex beads with a diameter of 20 nm (20 nm LB) from lysosomes into phagosomes containing HKLM. Already from 10 min after co-incubation on, the percentage of HKLM colocalizing with 20 nm LB was significantly lower in ASMase$^{-/-}$ macrophages than in wt macrophages (Fig. 8C).
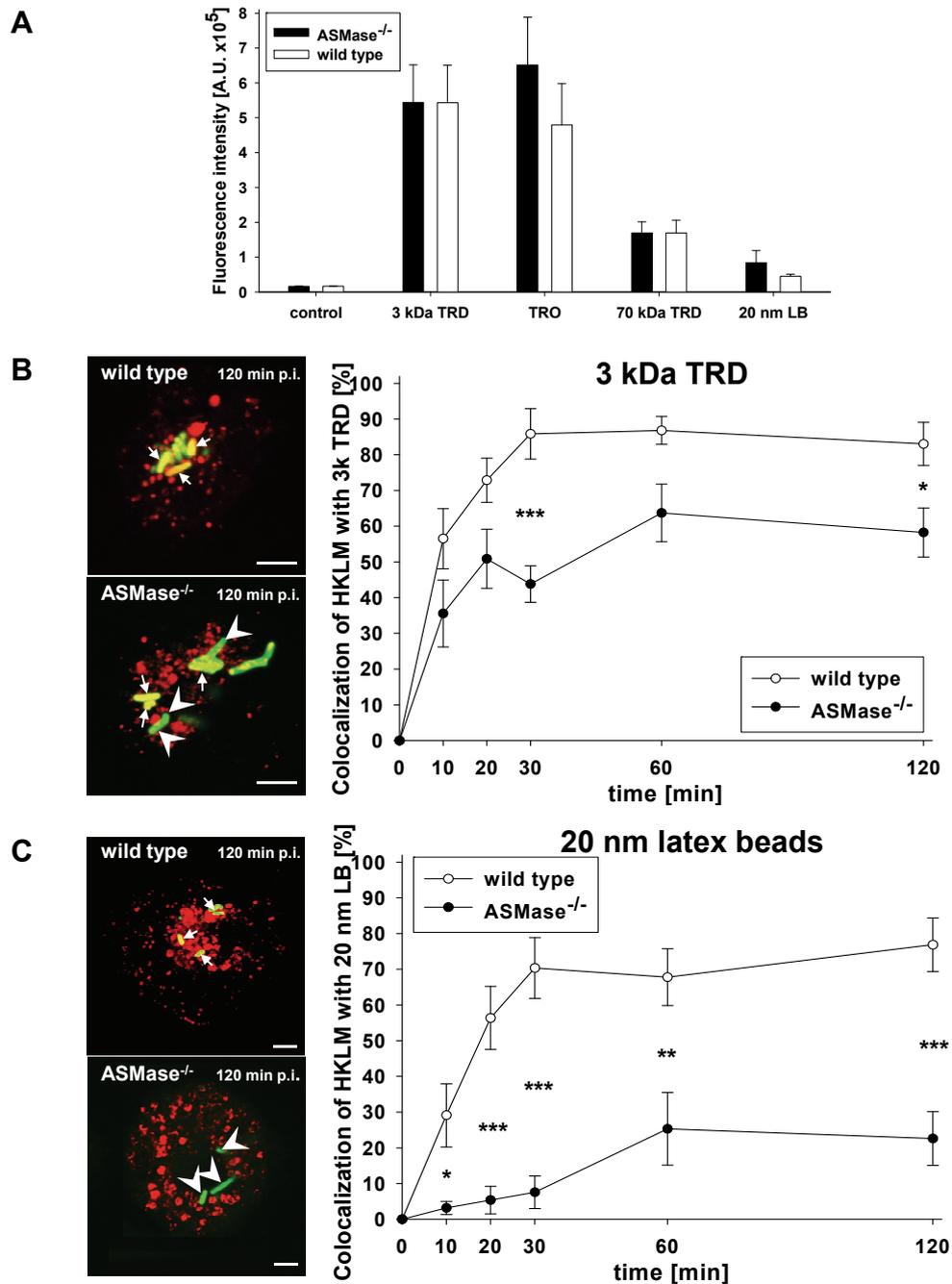
Fig. 8: Impaired transfer of fluid phase markers from lysosomes into phagosomes containing HKLM in ASMase^{-/-} macrophages.

Lysosomes in proteose peptone-elicited peritoneal macrophages from ASMase^{-/-} or wt littermates were pulse/chase-labelled with diverse fluorescent fluid phase markers. (A) Fluorometric quantification of the uptake of 3 kDa dextran conjugated to TexasRed (3 kDa TRD), 45 kDa ovalbumin conjugated to TexasRed (TRO), 70 kDa dextran conjugated to TexasRed (70 kDa TRD) or latex beads with a diameter of 20 nm (20 nm LB) into immunomagnetically-enriched ASMase^{-/-} and wt macrophages. Results of three independent experiments are shown as mean +/- s.e.

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(Legend of Fig. 8 / continued)

(B, C) ASMase$^{-/-}$ and wt macrophages were co-incubated at a MOI-equivalent of 5 with FITC-labelled HKLM. At the indicated times after co-incubation, phagocytosed HKLM were analysed for colocalization with (B) 3kDa TRD or (C) 20 nm LB by confocal laser scanning microscopy. Representative micrographs indicating colocalization (arrows) or non-colocalization (arrow heads) of HKLM with fluid phase marker molecules at 120 min after co-incubation are shown as merged images. Bars are 4 µm. At each time point, a minimum of 100 phagosomes from three independent experiments was analysed. Percentage of Listeria per macrophage colocalizing with fluid phase marker molecules are shown as mean +/- s.e. The statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA. P-values are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Strikingly, the transfer of 20 nm LB in ASMase$^{-/-}$ macrophages was more impaired than the transfer of 3 kDa TRD (compare Fig. 8B and 8C). Thus, we hypothesised that the transfer of lysosomal cargo into phagosomes is more impaired for cargo of high molecular weight. To test this hypothesis, we investigated the transfer of lysosomal fluid phase markers of graded size into phagosomes containing *L. monocytogenes*.

In wt macrophages, over 80% of the phagosomes containing HKLM were positive for 3 kDa TRD, 45 kDa ovalbumin conjugated to TexasRed (TRO; about 6 nm in diameter), dextran of 70 kDa MW conjugated to TexasRed (70 kDa TRD; about 12 nm in diameter) or 20 nm LB at 120 min after co-incubation (Fig. 8D). In sharp contrast, the percentage of phagosomes which contained both HKLM and fluid phase marker molecules decreased gradually with increasing size of the fluid phase markers in ASMase$^{-/-}$ macrophages.

Similar results were obtained using viable but apathogenic strains of *L. monocytogenes* which are deficient in listeriolysin O (Δ*hly-L. monocytogenes)* or the main transcriptional regulator of listerial virulence factors, prfA (Δ*prfA-L. monocytogenes*) (Peters, Domann et al. 2003). In wt macrophages, 80-90% of either Δ*hly-* or Δ*prfA-L. monocytogenes* colocalized with 3 kDa TRD as well as with 20 nm LB at 120 min p.i. (Fig. 8D). In sharp contrast, only 60-70% of either Δ*hly-* or Δ*prfA-L. monocytogenes* colocalized with 3 kDa TRD in ASMase$^{-/-}$ macrophages while only about 30% colocalized with 20 nm LB.

These data show that, in ASMase$^{-/-}$ macrophages, (i) fusion of late phagosomes with lysosomes is significantly impaired and (ii) transfer of lysosomal fluid phase markers into phagosomes is more impaired with increasing size of the marker.
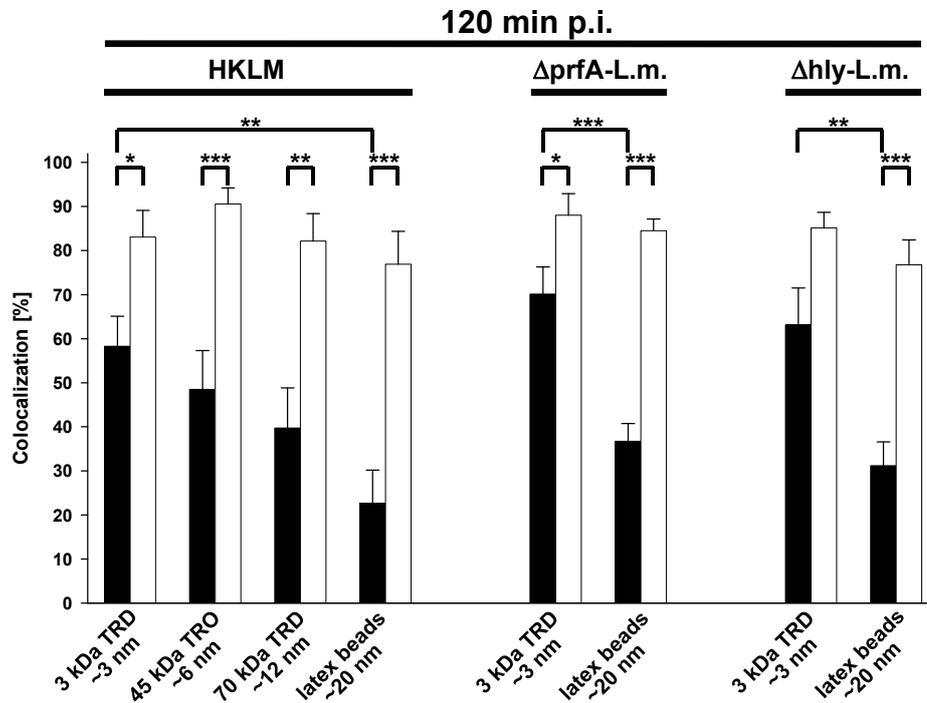

Fig. 9: Transfer of fluid phase markers from lysosomes into phagosomes containing *L. monocytogenes* in ASMase$^{-/-}$ macrophages is more impaired with increasing size of the cargo.

Compilation of the colocalization of HKLM or viable Δ*hly*- or Δ*prfA*-*L. monocytogenes* with 3 kDa TRD (diameter of about 3 nm), 45 kDa TRO (diameter of about 6 nm), 70 kDa TRD (diameter of about 12 nm) or 20 nm LB (diameter of about 20 nm) in ASMase$^{-/-}$ (black bars) or wild type (white bars) macrophages at 120 min after co-incubation. At each time point, a minimum of 100 phagosomes from three independent experiments was analysed. Percentage of Listeria per macrophage colocalizing with fluid phase marker molecules are shown as mean +/- s.e. The statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA. P-values are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

The transfer of artificial lysosomal cargo might not reflect the situation of endogenous cargo. To investigate whether beyond the transfer of lysosomal fluid phase markers also the transfer of endogenous lysosomal cargo is impaired in ASMase$^{-/-}$ cells, we investigated the transfer of lysosomal proteases into phagosomes containing *L. monocytogenes*. Phagosomes containing Δ*prfA*-
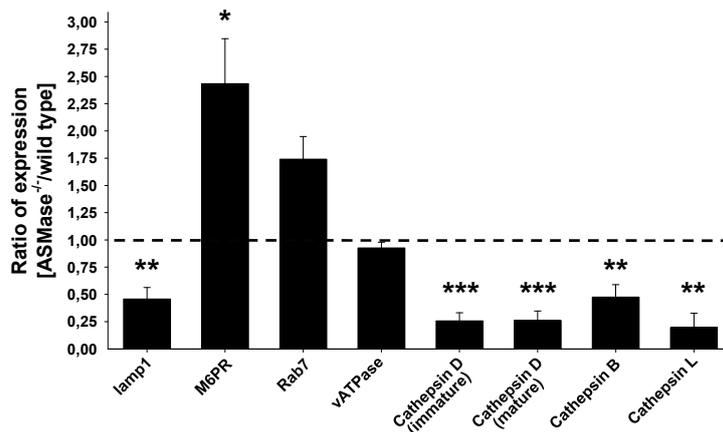
L. monocytogenes were prepared from mouse embryonic fibroblasts (MEF) or from bone marrow-derived macrophages (BMDM) 2 h p.i. and analysed by Western blotting. Phagosomes containing Δ*prfA*-*L. monocytogenes* from both ASMase$^{-/-}$ MEF and ASMase$^{-/-}$ BMDM contained significantly more M6PR and Rab7 but significantly less Lamp1 than wt controls at 2 h p.i. (Fig. 10A, left panel and Fig. 10B). The E-subunit of the vesicular proton pump (vATPase), however, was expressed at similar levels on phagosomes from ASMase$^{-/-}$ and wt MEF or BMDM.

Most importantly, phagosomes containing Δ*prfA*-*L. monocytogenes* contained significantly less of the lysosomal proteases cathepsin D (both the immature and the mature form), cathepsin B and cathepsin L in ASMase$^{-/-}$ MEF or BMDM than wt controls (Fig. 10A, right panel and Fig. 10B). Cell lysates of both ASMase$^{-/-}$ and wt MEF or BMDM, however, contained equal amounts of any of the above listed cathepsins (Fig. 10C). Thus, despite the presence of normal amounts of proteases, the transfer of lysosomal proteases into phagosomes containing *L. monocytogenes* is significantly impaired in ASMase$^{-/-}$ cells.

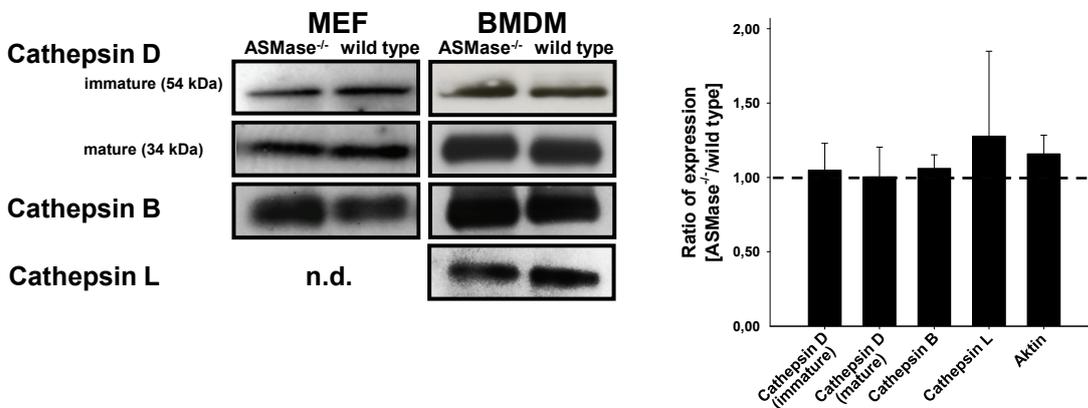**A**   **Phagosomes containing ΔprfA-L.m. 120 min p.i.**

MEF	BMDM
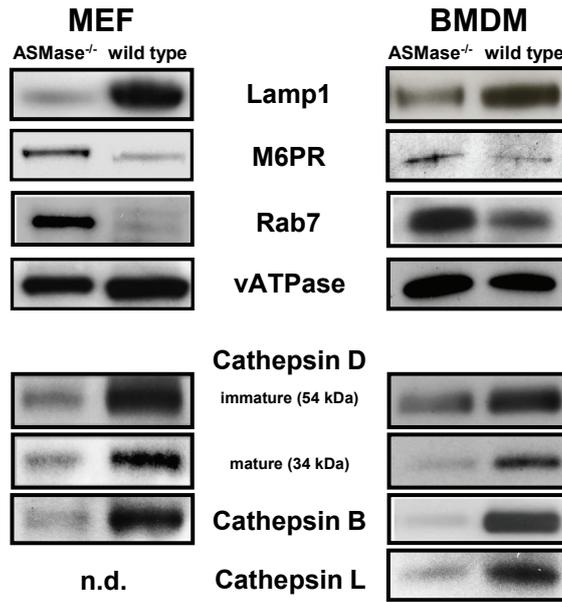
B

C   **Cathepsin expression in MEF and BMDM**

(Legend on following page)

Fig. 10: Impaired transfer of lysosomal proteases into phagosomes containing *L. monocytogenes* in ASMase$^{-/-}$ cells.

(A) Phagosomes containing ΔprfA-*L. monocytogenes* were prepared from ASMase$^{-/-}$ or wt MEF and BMDM 2 h p.i. and analysed for presence of Lamp1, M6PR, Rab7, vATPase, cathepsin D, cathepsin B and cathepsin L (not detectable (n.d.) in MEF) by Western blot. The lanes were loaded with equal amounts of protein as determined by BCA protein assay. (B) Densidometry of bands specific for the indicated proteins expressed as ratio of ASMase$^{-/-}$ to wild type. Data from at least three independent experiments are shown as mean +/- s.e. The statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA. P-values are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$). (C) Lysates of ASMase$^{-/-}$ or wt MEF and BMDM were analysed for contents of cathepsin D, cathepsin B, cathepsin L and actin by Western blot. Expression in ASMase$^{-/-}$ compared to wild type cells was determined by densidometry of bands specific for the indicated proteins. Data from at least three independent experiments are shown as mean +/- s.e.

To investigate whether the impaired transfer of lysosomal proteases results in reduced proteolytic activity in ASMase$^{-/-}$ macrophages, we analysed the proteolytic degradation of two model protein substrates, hen egg lysozyme (HEL) and horseradish peroxidase (HRP). HEL is known to be more susceptible to proteolytic degradation than HRP (Trombetta, Ebersold et al. 2003). Similar amounts of both protein substrates were endocytosed by ASMase$^{-/-}$ and wt macrophages (Fig. 11 A; t = 0 h). HEL was rapidly degraded by both ASMase$^{-/-}$ and wt macrophages (Fig. 11 A). In contrast, HRP was degraded less efficiently in ASMase$^{-/-}$ macrophages than in wt macrophages indicating that ASMase$^{-/-}$ macrophages are impaired in proteolytic degradation of select substrates.

As non-lysosomal proteases may contribute to the above described degradation of HEL and HRP (Claus, Jahraus et al. 1998), we determined the proteolytic degradation of a peptide substrate which is specifically degraded by the lysosomal proteases cathepsin B and L (Yates, Hermetter et al. 2005). After IgG-mediated phagocytosis of latex beads coated with the peptide substrate, unquenching of peptide substrate fluorescence by cathepsin B- and L-mediated proteolysis was analysed by quantitative fluorometry. From t = 15 min on, peptide substrate fluorescence increased much slower in ASMase$^{-/-}$ than in wt macrophages indicating impaired proteolytic activity of lysosomal cathepsins in phagosomes of ASMase$^{-/-}$ macrophages (Fig. 11 B).
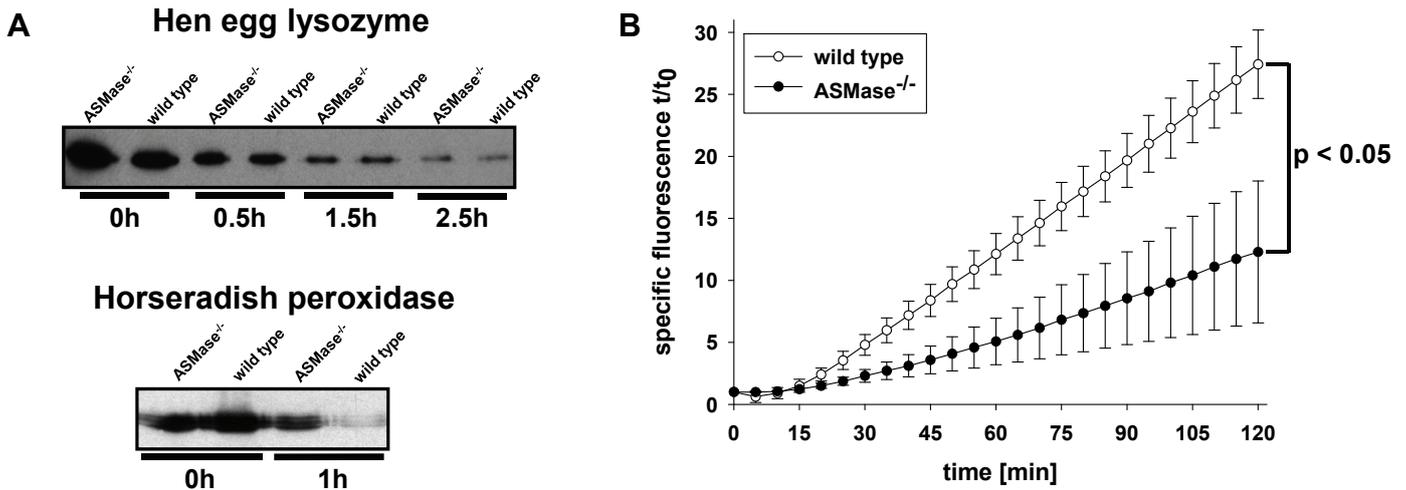
A   **Hen egg lysozyme**

0h   0.5h   1.5h   2.5h

Horseradish peroxidase

0h   1h

B

p < 0.05

Fig. 11: Impaired proteolytic activity in ASMase$^{-/-}$ macrophages.

(A) Proteose peptone-elicited peritoneal macrophages from ASMase$^{-/-}$ or wt littermates were pulse-chase incubated with hen egg lysozyme (HEL) or horseradish peroxidase (HRP). At the indicated times after co-incubation, macrophages were lysed and contents of HEL and HRP analysed by Western blot. (B) Kinetics of intraphagosomal proteolytic activity in immunomagnetically enriched proteose peptone-elicited ASMase$^{-/-}$ and wt macrophages. Dequenching of peptide substrate fluorescence after specific hydrolysis by lysosomal cathepsins B and L was determined by quantitative fluorometry as described in the Methods section (2.2.12). Data from three independent experiments are shown as mean +/- s.e. The statistical significance of the difference between ASMase$^{-/-}$ and wild type was determined by one-way ANOVA.

Taken together, our data show that fusion of late phagosomes with lysosomes is impaired which results in impaired transfer of bactericidal lysosomal proteases into phagosomes containing *L. monocytogenes*.

4 Discussion

ASMase$^{-/-}$ mice are highly susceptible to infection with *L. monocytogenes* because ASMase$^{-/-}$ macrophages cannot kill phagocytosed *L. monocytogenes* (Utermohlen, Karow et al. 2003). The precise ASMase-dependent antibacterial mechanism, however, remained elusive. Here, we show that ASMase is required for efficient phago-lysosomal fusion and consequently for transfer of bactericidal acid hydrolases from lysosomes into phagosomes.

ASMase has been shown to be essential for entry of *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* into human epithelial cells (Grassme, Gulbins et al. 1997; Grassme, Jendrossek et al. 2003). In contrast, uptake of *Escherichia coli* into murine peripheral blood leukocytes does not depend on ASMase (Utermohlen, Karow et al. 2003). Here, we show that ASMase$^{-/-}$ peritoneal macrophages phagocytose *L. monocytogenes* as efficiently as wt macrophages. Thus, ASMase is not required for phagocytosis of *L. monocytogenes* by macrophages.

After phagocytosis, *L. monocytogenes* rapidly escaped from phagosomes, recruited actin and massively proliferated in ASMase$^{-/-}$ but not in wt macrophages. This is consistent with our previous report that *L. monocytogenes* escapes bactericidal effector mechanisms in ASMase$^{-/-}$ macrophages (Utermohlen, Karow et al. 2003).

Oxidative bactericidal effector mechanisms which comprise both reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) have been shown to be crucial to retain virulent *L. monocytogenes* in phagosomes (Myers, Tsang et al. 2003). Although the generation of ROI has been reported to be significantly decreased after siRNA-knock down of ASMase in coronary arterial endothelial cells (Zhang, Yi et al. 2007) we found ASMase$^{-/-}$ and wt macrophages to generate similar amounts of both ROI and RNI after stimulation with PMA or HKLM, respectively. Furthermore, the catalytic p22^{phox} subunit of the NADPH oxidase complex was recruited to phagosomes containing *L. monocytogenes* with similar kinetics in ASMase$^{-/-}$ and wt macrophages. These data confirm and extend previous observations that ASMase$^{-/-}$ peritoneal exudate cells and peripheral blood lymphocytes produce normal amounts of ROI and RNI after stimulus with TNF, LPS or *Salmonella typhimurium* (Utermohlen, Karow et al. 2003; McCollister, Myers et al.

2007). Together, these data indicate that ASMase is not required for activity of oxidative effector mechanisms in macrophages.

The lack of ASMase-dependent alterations in oxidative bactericidal effector mechanisms shifted our attention to a possible role of ASMase in non-oxidative bactericidal effector mechanisms. In recent years, non-oxidative bactericidal effector mechanisms have been shown to contribute substantially to bactericidal activity (Reeves, Lu et al. 2002; Prada-Delgado, Carrasco-Marin et al. 2005; Segal 2005; del Cerro-Vadillo, Madrazo-Toca et al. 2006). Particularly acid hydrolases like the lysosomal protease cathepsin D have been shown to be important for the inactivation of *L. monocytogenes* in macrophages (del Cerro-Vadillo, Madrazo-Toca et al. 2006). As these acid hydrolases are transferred from lysosomes into phagosomes at the end of the phagosomal maturation process (Haas 2007) we next investigated whether maturation of phagosomes depends on ASMase.

Maturation of phagosomes containing *L. monocytogenes* was not altered in ASMase$^{-/-}$ macrophages at the early or late phagosomal stages as indicated by normal acquisition of three independent marker molecules: (i) p22^{phox}, (ii) M6PR and (iii) Rab7. The p22^{phox} subunit of the NADPH oxidase complex is an integral membrane protein of both the plasma membrane and vesicles fusing with phagosomes very early after generation of phagosomes (Ginsel, Onderwater et al. 1990; Nauseef 2004). The prompt colocalization of p22^{phox} with *L. monocytogenes* in both ASMase$^{-/-}$ and wt macrophages indicates that internalization of *L. monocytogenes* and the first steps of the maturation of phagosomes containing *L. monocytogenes* do not depend on ASMase. Maturation of phagosomes into late phagosomes is characterized by acquisition of M6PR and Rab7 (Vieira, Botelho et al. 2002; Scott, Botelho et al. 2003; Haas 2007). Phagosomes containing *L. monocytogenes* acquired both M6PR and Rab7 with similar kinetics in ASMase$^{-/-}$ and wt macrophages. This indicates that maturation until the late phagosomal stage is not impaired in ASMase$^{-/-}$ macrophages.

Maturation of late phagosomes into phagolysosomes is characterized by the loss of M6PR and Rab7 and the acquisition of the (phago)lysosomal marker Lamp1 (Vieira, Botelho et al. 2002; Scott, Botelho et al. 2003; Haas 2007). Furthermore, intraphagosomal pH drops from between 6.0 and 5.0 in late phagosomes to between 5.0 and 4.0 in phagolysosomes (Haas 2007). Surprisingly, both M6PR and Rab7 remained present on phagosomes containing *L. monocytogenes* in ASMase$^{-/-}$ but not

wt macrophages. This was paralleled by an impaired acquisition of Lamp1 by phagosomes containing *L. monocytogenes* in ASMase^{-/-} macrophages. Furthermore, phagosomes containing *L. monocytogenes* did not acidify below a pH of about 5 in ASMase^{-/-} macrophages while pH dropped to about 4 in phagosomes of wt macrophages. Together, these data indicate that the maturation of late phagosomes containing *L. monocytogenes* into phagolysosomes is impaired in ASMase^{-/-} macrophages.

L. monocytogenes has been shown to actively delay phagosomal maturation in J774E and CHO cells (Alvarez-Dominguez, Barbieri et al. 1996; Alvarez-Dominguez, Roberts et al. 1997; Prada-Delgado, Carrasco-Marin et al. 2005; Alvarez-Dominguez, Madrazo-Toca et al. 2008). In ASMase^{-/-} macrophages, however, maturation of late phagosomes into phagolysosomes was impaired independently of the viability or the virulence of *L. monocytogenes*. Similar results were obtained with viable and heat-killed wt *L. monocytogenes* as well as with avirulent deletion mutants of *L. monocytogenes* lacking either listeriolysin O (LLO) (Δhly-*L. monocytogenes*) or the main transcriptional regulator of listerial virulence factors, prfA (ΔprfA-*L. monocytogenes*). These data indicate that maturation of late phagosomes into phagolysosomes is impaired in ASMase^{-/-} macrophages because of a genuine cellular defect rather than by active interference of viable *L. monocytogenes*.

During maturation of late phagosomes into phagolysosomes, lysosomal cargo is transferred into phagosomes by phago-lysosomal fusion (Vieira, Botelho et al. 2002; Scott, Botelho et al. 2003; Haas 2007). Therefore, transfer of fluid phase markers from pre-labelled lysosomes into phagosomes is a direct functional indicator of phago-lysosomal fusion (Desjardins, Celis et al. 1994; Desjardins, Huber et al. 1994; Jahraus, Tjelle et al. 1998; Ward, Pevsner et al. 2000). In ASMase^{-/-} macrophages, the transfer of fluid phase markers from lysosomes into phagosomes containing *L. monocytogenes* was significantly impaired proving functionally a defect in phago-lysosomal fusion. Transfer of lysosomal cargo was impaired in ASMase^{-/-} macrophages for cargo of various chemical composition or size of the cargo: transfer of polysaccharides (dextrans), proteins (ovalbumin) and polystyrene beads (latex beads) was impaired similarly and even transfer of small 3 kDa dextran molecules was significantly reduced. Because the transfer of artificial lysosomal cargo might not reflect the situation of endogenous cargo we investigated the transfer of endogenous lysosomal cargo molecules. Transfer of the lysosomal cathepsins D, B and L into

phagosomes containing *L. monocytogenes* was impaired in ASMase^{-/-} macrophages as compared to wt macrophages. This shows that transfer of endogenous cargo from lysosomes into phagosomes is impaired in ASMase^{-/-} macrophages. Consequently, proteolytic activity was significantly reduced in phagosomes of ASMase^{-/-} macrophages. Taken together, these data show that the impaired phago-lysosomal fusion in ASMase^{-/-} macrophages not only impairs the transfer of exogenous fluid phase markers but also the transfer of endogenous lysosomal proteases.

Particularly transfer of cathepsin D into phagosomes contributes to listeriocidal activity of macrophages because mature cathepsin D has been shown to degrade listeriolysin O (del Cerro-Vadillo, Madrazo-Toca et al. 2006). Although maturation of inactive pro-cathepsin D into active cathepsin D has been reported to depend on ASMase-derived ceramide (Heinrich, Wickel et al. 1999; Heinrich, Wickel et al. 2000) cell lysates of ASMase^{-/-} and wt BMDM and MEF contained similar amounts of mature cathepsin. However, the transfer of both immature and mature cathepsin D into phagosomes containing *L. monocytogenes* was significantly reduced in ASMase^{-/-} BMDM and MEF. Together, our data indicate that ASMase is required for efficient phago-lysosomal fusion and thereby for transfer of bactericidal acid hydrolases from lysosomes into phagosomes containing *L. monocytogenes*.

How does ASMase contribute to phago-lysosomal fusion? SMases directly influence the fusogenicity of biomembranes (Zha, Pierini et al. 1998; Goni and Alonso 2000; Holopainen, Angelova et al. 2000; Goni and Alonso 2002). For example, the hydrolysis of sphingomyelin into ceramide by SMases *in vitro* promotes liposome fusion (Ruiz-Arguello, Basanez et al. 1996; Basanez, Ruiz-Arguello et al. 1997; Hinkovska-Galcheva, Boxer et al. 1998; Oorni, Hakala et al. 1998; Ruiz-Arguello, Goni et al. 1998). ASMase is required for exocytosis of cytotoxic effector molecules by fusion of cytotoxic granules with the plasma membrane in CD8+ cytotoxic T lymphocytes (Utermohlen, Herz et al. 2008). Furthermore, treatment with exogenous ceramide promotes maturation of phagosomes arrested by pathogenic mycobacteria in early phagosomal stage. This ceramide-induced maturation leads to killing of the mycobacteria in phagolysosomes which shows the relevance of ceramide for antimicrobial activity of macrophages (Anes, Kuhnel et al. 2003).

These effects can be explained by the biophysical properties of ceramide (Fig. 12 A). SMases remove the large polar head group phosphorylcholine from sphingomyelin resulting in generation of ceramide. Because of its small head group

ceramide is cone-shaped. Within biomembranes, this cone-shape induces bending of the membrane towards the small head group of ceramide (Zha, Pierini et al. 1998).
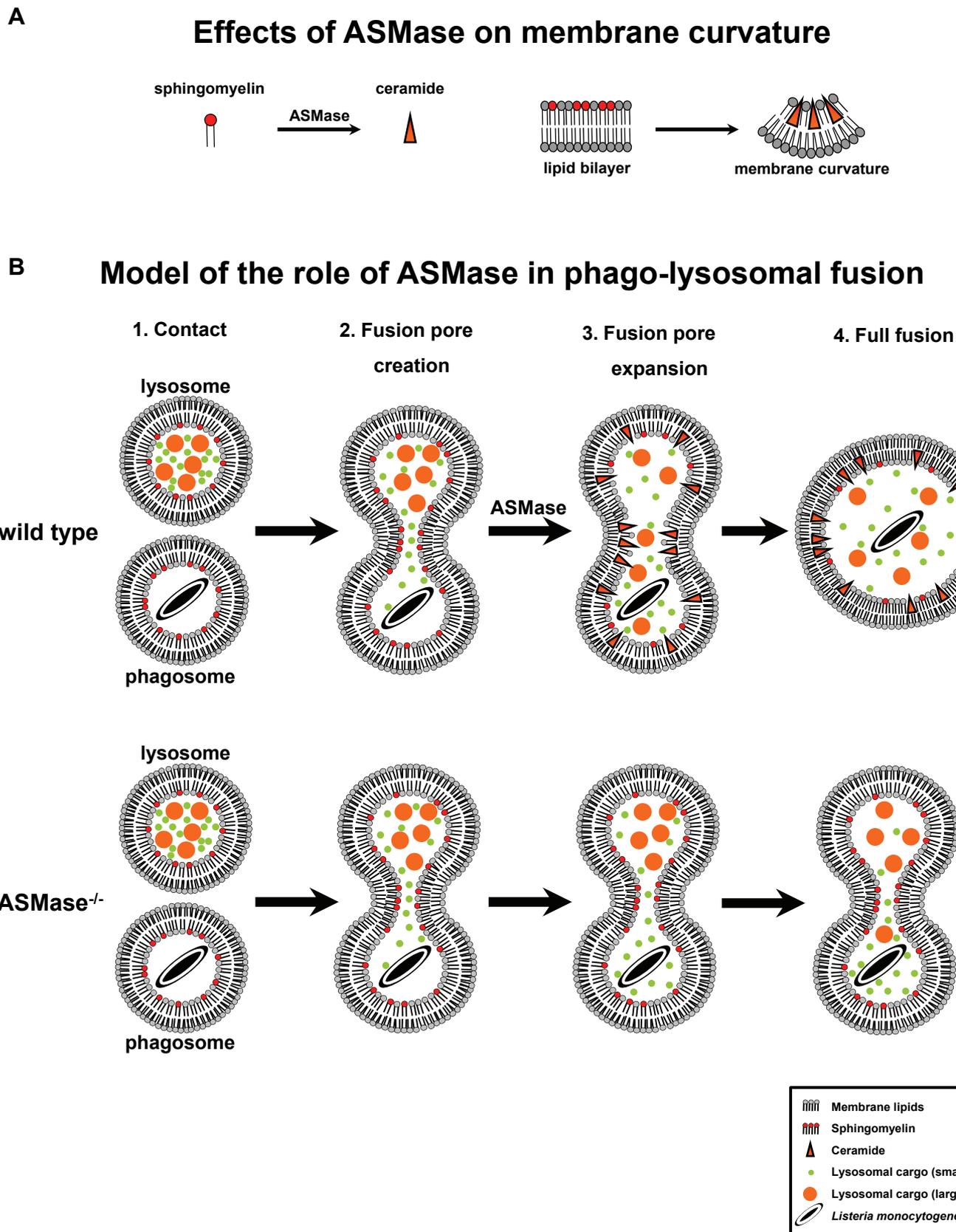
A

Effects of ASMase on membrane curvature

B

Model of the role of ASMase in phago-lysosomal fusion

Fig. 12: Model of the role of ASMase during phago-lysosomal fusion

(A) Removal of the large polar head group phosphorylcholine from sphingomyelin by ASMase generates cone-shaped ceramide. In biomembranes, cone-shaped ceramide

(Legend of Fig. 12 / continued)

induces bending towards its small head group thereby generating negative membrane curvature.

(B) Following (1) contact of phagosome and lysosome, (2) an initial fusion pore is formed and (3) subsequently expanded until (4) full fusion of both vesicles in wild type cells. During formation of the initial fusion pore, local membrane curvature in the region of the fusion pore changes from negative to strongly positive. By hydrolyzing sphingomyelin into cone-shaped ceramide, ASMase generates negative membrane curvature in this region and thereby reduces the positive membrane curvature which leads to fusion pore expansion. In ASMase$^{-/-}$ macrophages, no ceramide is generated and consequently the initial fusion pore is not expanded.

Thus, the membrane becomes concave, or negatively curved towards the leaflet containing ceramide. Membrane curvature has been discussed to play a pivotal role in vesicle fusion, particularly during the formation and expansion of fusion pores (Chernomordik and Kozlov 2003).

Fusion pores are aqueous channels connecting the lumina of fusing vesicles after merging of the lipid bilayers. During full fusion of vesicles, the narrow initial fusion pore is expanded until the vesicles fully merge. During kiss-and-run fusion, however, a transient fusion pore of limited size allows only limited exchange of cargo during the kiss-phase (Desjardins 1995; Bright, Gratian et al. 2005). After resealing of the fusion pore, the vesicles separate again (run-phase). During kiss-and-run fusion, the fusion pores of limited size and lifespan favour the transfer of small cargo molecules over larger molecules whereas during full fusion cargo of any size is transferred (Kishimoto, Kimura et al. 2006). Thus, fluid phase markers of distinct size allow differentiation between full fusion and kiss-and-run fusion (Wang and Goren 1987; Desjardins 1995; Duclos, Diez et al. 2000; Takahashi, Kishimoto et al. 2002; Kishimoto, Kimura et al. 2006). It has been shown that full fusion occurs if the fusion pore is expanded beyond a critical diameter of 12 nm. Otherwise, the fusion pore collapses and is resealed resulting in kiss-and-run fusion (Takahashi, Kishimoto et al. 2002; Kishimoto, Kimura et al. 2006).

In ASMase$^{-/-}$ macrophages, transfer of lysosomal cargo into phagosomes was the more impaired the larger the size of the cargo molecules. In wt macrophages, in

contrast, transfer was independent of the cargo size. This indicates that in ASMase$^{-/-}$ macrophages cargo is transferred from lysosomes into phagosomes via fusion pores of limited size and/or lifespan like during kiss-and-run fusion, suggesting that ASMase is required for fusion pore expansion during phago-lysosomal fusion.

The exact driving forces of fusion pore expansion are currently unknown (Jackson and Chapman 2006), however, lipids altering membrane curvature have been discussed to play a direct role (Chernomordik and Kozlov 2003).

Fig. 12 B illustrates a mechanism by which ASMase could contribute to fusion pore expansion by altering the local membrane curvature. Membrane curvature of the intraluminal leaflet of vesicles is negative. However, when the initial fusion pore is formed, membrane curvature locally changes into strongly positive. As positive membrane curvature stimulates activity of ASMase by providing better access to the cleavage site of sphingomyelin (Linke, Wilkening et al. 2001), sphingomyelin hydrolysis into ceramide in the positively curved region of the fusion pore could reduce local positive curvature leading to fusion pore expansion.

Taken together, the data of this study show that ASMase is required for fusion of lysosomes with phagosomes containing *L. monocytogenes*. Correspondingly, ASMase$^{-/-}$ macrophages cannot kill phagocytosed *L. monocytogenes* despite normal activity of oxidative effector mechanisms because the transfer of bactericidal acid hydrolases into phagosomes containing *L. monocytogenes* by phago-lysosomal fusion is strongly impaired.

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6 Summary

The acid sphingomyelinase (ASMase) hydrolyzes sphingomyelin, a major component of the outer leaflet of biomembranes. ASMase is present in endosomes, phagosomes and lysosomes, i.e. the compartments which are most important for antimicrobial activity of macrophages. In contrast to macrophages of wild type mice, acid sphingomyelinase-deficient (ASMase$^{-/-}$) macrophages are not able to restrict intracellular growth of *Listeria monocytogenes* (*L. monocytogenes*). In ASMase$^{-/-}$ macrophages, phagocytosed *L. monocytogenes* escape from phagosomes, recruit actin and massively proliferate in the cytosol despite normal activity of oxidative bactericidal effector mechanisms. Kinetic analysis of phagosomal maturation revealed that phagosomes containing either viable *L. monocytogenes*, heat-killed *L. monocytogenes* (HKLM) or the avirulent Δhly- or ΔprfA-mutants of *L. monocytogenes* fail to mature into bactericidal phagolysosomes in ASMase$^{-/-}$ macrophages. Phagosomes containing *L. monocytogenes* remained positive for the late phagosomal markers M6PR and Rab7 but acquired significantly less Lamp1. Furthermore, phagosomes containing HKLM did not acidify properly in ASMase$^{-/-}$ macrophages. Transfer of fluid phase markers from lysosomes into phagosomes containing HKLM was significantly impaired in ASMase$^{-/-}$ macrophages indicating impaired phago-lysosomal fusion. Moreover, phagosomes containing *L. monocytogenes* in ASMase$^{-/-}$ macrophages acquired significantly less of the bactericidal cathepsins D, B and L resulting in significantly reduced proteolytic activity. Together, these data indicate that ASMase is necessary for fusion of late phagosomes with lysosomes and thus for the generation of bactericidal phagolysosomes.

7 Zusammenfassung

Die saure Sphingomyelinase (ASMase) hydrolysiert Sphingomyelin, einen wichtigen Bestandteil des äußeren Blattes von Biomembranen. Die ASMase ist in Endosomen, Phagosomes und Lysosomen und damit in den Kompartimenten, die für die antimikrobielle Aktivität von Makrophagen am wichtigsten sind, präsent. Im Gegensatz zu Makrophagen aus Wildtyp-Mäusen können ASMase-defiziente (ASMase$^{-/-}$) Makrophagen das intrazelluläre Wachstum von *Listeria monocytogenes* (*L. monocytogenes*) nicht unterbinden. In ASMase$^{-/-}$ Makrophagen entkommen phagozytierte *L. monocytogenes* aus dem Phagosom, rekrutieren Aktin und vermehren sich massiv im Zytosol, obwohl die Aktivität der oxidativen bakteriziden Effektormechanismen normal ist. Die Analyse der Kinetik der phagosomalen Reifung zeigte, dass Phagosomen, die entweder lebende *L. monocytogenes*, hitzegetöte *L. monocytogenes* (HKLM) oder die avirulenten Δhly- oder ΔprfA-Mutanten von *L. monocytogenes* enthalten, in ASMase$^{-/-}$ Makrophagen nicht zu bakteriziden Phagolysosomen reifen. *L. monocytogenes*-enthaltende Phagosomen blieben in ASMase$^{-/-}$ Makrophagen positiv für die spät-endosomalen Marker M6PR und Rab7 und akquirierten signifikant weniger Lamp1. Weiterhin, wurden HKLM-enthaltende Phagosomen in ASMase$^{-/-}$ Makrophagen signifikant schlechter angesäuert. Der Transfer von Flüssigphasenmarkern aus Lysosomen in HKLM-enthaltende Phagosomen war in ASMase$^{-/-}$ Makrophagen signifikant gestört, was auf eine gestörte phago-lysosomale Fusion hinweist. Weiterhin acquirierten *L. monocytogenes*-enthaltende Phagosomen in ASMase$^{-/-}$ Makrophagen signifikant weniger Cathepsin D, B und L, was zu einer signifikant verringerten proteolytischen Aktivität führte. Zusammenfassend deuten diese Daten darauf hin, dass die ASMase notwendig für die Fusion von späten Phagosomen mit Lysosomen und damit für die Generierung von bakteriziden Phagolysosomen ist.

9 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Arbeit ist von Herrn Prof. Dr. Martin Krönke betreut worden.

Teilpublikationen

Michael Schramm, Jasmin Herz, Albert Haas, Martin Krönke, Olaf Utermöhlen. **Acid sphingomyelinase is required for efficient phago-lysosomal fusion.** *Cellular Microbiology* (2008); 10(9), 1839-1853

Olaf Utermöhlen, Jasmin Herz, Michael Schramm, Martin Krönke. **Fusogenicity of membranes: The impact of acid sphingomyelinase on innate immune responses.** *Immunobiology* (2008); 213(3-4):307-14

Köln, den 23.09.08

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