Vacuolar ATPase in Phagosome-Lysosome Fusion

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1 Background: The vacuolar H⁺-ATPase complex is thought to contribute to membrane fusion.

Results: v-ATPase complex knock-out experiments in mice revealed that its absence does not affect phagosome-lysosome fusion.

Conclusion: Participation of v-ATPase in phagosome-lysosome fusion is unlikely.

Significance: Fusion between lysosomes/late endosomes and phagosomes is not controlled by the v-ATPase.

The vacuolar H⁺-ATPase (v-ATPase) complex is instrumental in establishing and maintaining acidification of some cellular compartments, thereby ensuring their functionality. Recently it has been proposed that the transmembrane V0 sector of v-ATPase and its α-subunits promote membrane fusion in the endocytic and exocytic pathways independent of their acidification functions. Here, we tested if such a proton-pumping independent role of v-ATPase also applies to phagosome-lysosome fusion. Surprisingly, endolysosomes in mouse embryonic fibroblasts lacking the V0 α3 subunit of the v-ATPase acidified normally, and endosome and lysosome marker proteins were recruited to phagosomes with similar kinetics in the presence or absence of the α3 subunit. Further experiments used macrophages with a knockdown of v-ATPase accessory protein 2 (ATP6AP2) expression, resulting in a strongly reduced level of the V0 sector of the v-ATPase. However, acidification appeared undisturbed, and fusion between late bead-containing phagosomes and lysosomes, as analyzed by electron microscopy, was even slightly enhanced, as was killing of non-pathogenic bacteria by V0 mutant macrophages. Pharmacologically neutralized lysosome pH did not affect maturation of phagosomes in mouse embryonic cells or macrophages. Finally, lacking the two large parts of the v-ATPase complex together by the drug saliphenylhalamide A did not inhibit in vitro and in cellulo fusion of phagosomes with lysosomes. Hence, our data do not suggest a fusion-promoting role of the v-ATPase in the formation of phagolysosomes.

A phagocytic compartment (phagosome) is formed when a particle is ingested through receptor-ligand interaction into a plasma membrane invagination. Newly formed phagosomes are not static compartments but rather acquire degradative and microbicidal properties through a complex series of interactions with endomembranes. This process, collectively termed phagosome maturation, culminates in the fusion of phagosomes with lysosomes, yielding a strongly acidic (pH 4.5–5.0) hybrid organelle enriched in hydrolytic enzymes and antimicrobial peptides that promotes killing and degradation of internalized microorganisms (1, 2). The main role of phagocytosis is the delivery of microbial invaders and apoptotic bodies to phagolysosomes. For most microbes the acidic, hydrolytically competent environment of the eventually formed phagolysosome is sufficient to kill them. The strong acidification of (phago-)lysosomes is a hallmark feature of the endocytic and phagocytic pathways and is generated by a large multiprotein complex, the vacular ATPase (v-ATPase) (3, 4). Acidification is required for trafficking of endosomes (5) and for killing of ingested microorganisms (6). The precise subcellular origin of all vesicles, which can deliver v-ATPases to phagosomes is, however, not clear. The v-ATPase complex is formed by at least 14 subunits that are organized in two large subparticles; of these, the V0 sector contains transmembrane proteins that form the proton channel, whereas the V1 sector is cytosolic and is responsible for the ATPase activity (7). How exactly the subunits are assembled within the endoplasmic reticulum and delivered to lysosomes is not understood. However, it has been shown that “accessory subunits,” such as the v-ATPase accessory protein 2 (ATP6AP2 (8)), are important and that a failure to properly assemble the V0 portion leads to its degradation. Hence, it is not surprising that mutated v-ATPase subunits

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The abbreviations used are: v-ATPase, vacular ATPase; ATP6AP2, ATPase, H⁺-transporting lysosomal accessory protein 2; EKO, conditional knock-out; EEA1, early endosome antigen 1; LAMP-1, lysosomal-associated membrane protein 1; LAMP-2, lysosomal-associated membrane protein 2; LBPs, latex bead-containing phagosomes; SalPhe, saliphenylhalamide A; FcγRII, Fc-γ receptor II; BMDM, bone marrow-derived macrophage; MEF, mouse embryonic fibroblast.
cause many severe illnesses such as the progression of cancer and bone disorder (9, 10).

In the past few years evidence has accumulated that the transmembrane V0 part of the v-ATPase, without participation of the V1 sector, can play active and critical roles in membrane fusion along the endocytic and exocytic pathways independent of its proton-translocating activities. Such evidence stems from experiments on synaptic vesicle exocytosis in Caenorhabditis (13), secretion in Drosophila (14), osteoclast fusion (15), and vesicle fusion in zebrafish microglia cells after ingestion of neuron-derived apoptotic bodies (16). The original observation of an acidification-independent role in membrane fusion arose from studies on yeast homotypic vacuole fusion (11, 12). However, a recent study using the same model organelle proposed a role for vacuolar acidification (17).

To address the question in how far fusion events depend on the presence of the v-ATPase in mammalian cells, we used the v-ATPase together did not affect phagosome maturation and their care.

**Experimental Procedures**

### Antibodies

The following antibodies were used for protein detection in Western blot and immunofluorescence analyses: anti-Atp6ap2 N-terminal (HPA003156, Sigma), anti-β-actin (A2066, Sigma), anti-EAA1 (#C45B10, Cell Signaling Technology or #AV30074, Sigma), anti-cathepsin D (clone sII-10, a kind gift from Dr. S. Höning, University Cologne, Germany), anti-cathepsin L (#AF1515, R&D Systems), anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, clone FL335, #sc-25778, Santa Cruz Biotechnology), anti-LAMP1 (1D4B, DSHB), anti-LAMP-2 (Abi93, DSHB), anti-Myc (clone 9B11, #2276, Cell Signaling Technology), anti-transferrin receptor (TIB-219, ATCC), anti-V0 a1 (18), anti-V0 a2 (#ab96803, Abcam), anti-V0 a3 (generous gift from Dr. T. Jentsch, FMP, Berlin, Germany), anti-V0 d1 (#18274–1–AP, Proteintech group), anti-V0 A (kindly supplied by Dr. Shoji Ohkuma, Kanazawa University, Japan (19), anti-V1 E1 (20), rabbit polyclonal antibody to bovine B-subunit of vacuolar ATPase (Yoshinori Moriyama, Okayama University, Japan), and anti-V1 B2 (clone D2F9R, #14617, Cell Signaling Technology). Secondary antibodies conjugated to horseradish peroxidase, Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 were purchased from Dianova and Life Technologies. An anti-V0 c antibody was raised in rabbit against a peptide corresponding to residues 332–345 of the murine protein (DPGYDSIYRTMTNQ, Pineda-abservice).

### Experimental Animals

Mice with loxP sites flanking exon 2 of the Atp6ap2 gene have been described previously (21). Atp6ap22lox/+ female mice were bred with male mice, expressing the Cre recombinase under the control of an inducible Mx1 promoter (22) or the LysM promoter (23) to yield Atp6ap22lox/+Cre+/+ mice. Further breeding with homozygous Atp6ap22lox/+Cre+/+ female mice resulted in Atp6ap22lox/+Cre+/+ or Atp6ap22lox/+Cre-/+ animals (conditional knock-out (cKO)). Littermates negative for Mx1-Cre or LysM-Cre, respectively, served as control (wild-type).

Cre expression was induced in 6-week-old Mx1-Cre transgenic and control mice by intraperitoneal injection of 3 doses of 250 µg of polynosinic-polycytidylic acid (Sigma) within 5 days. Mice were kept for a further 10 days and sacrificed for experimental analysis. When required, intraperitoneal injection of 0.5 ml of 4% (w/v) Brewer’s thiglycollate solution (Difco/BD Biosciences) was performed to enrich peritoneal macrophages, and cells were harvested by peritoneal lavage 3 days later.

We are grateful to Dr. Uwe Kornak, Charité Berlin, Germany, for providing mice carrying the osteosclerotic mutation (oc/oc) in the Tcrg1 locus (24). All animal experiments were conducted in agreement with local guidelines for the use of animals and their care.

### Cell Lines and Primary Cell Culture

All cell types were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 4 mM l-glutamine and 4.5 g/liter glucose (PAA Laboratories or Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, PAA Laboratories or Biochrom AG). For maintenance of primary cells, 100 units/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories or Sigma) were added to the growth media. Cultures were grown at 37°C in a humidified 5% CO2 atmosphere condition unless stated otherwise.

Murine embryonic fibroblast were generated from 13–14-day-old embryos of breeding pairs yielding Tcrg1+/+/ (a3-deficient) and Tcrg1+/+ (wild-type) MEFs. Embryos were decapitated, and inner organs were removed before single cells were obtained by trypsin digest. All MEF lines were immortalized after three-four passages by transfection with the SV40 Large T antigen. Where indicated, MEFs were further cotransfected with Fc-γ receptor II (FcγRII; kind gift of Dr. Sergio Grinstein) and pcDNA4/TO (Addgene) and selected for stable protein expression in the presence of 250 µg/ml zeocin (InvivoGen). MEFs deficient for cathepsin D (CtsD−/−) and cathepsin L (CtsL−/−) have been described before (25, 26).

Murine primary peritoneal macrophages were collected by peritoneal lavage with 8 ml of ice-cold phosphate-buffered saline (PBS), centrifuged at 210 x g for 10 min at 4 °C, and resuspended in DMEM with FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin for plating. Non-adherent cells were removed after 3 h incubation at 37 °C and 5% CO2, and experiments were conducted the following day.
v-ATPase in Membrane Fusion

To obtain bone marrow-derived macrophages (BMDMs), long bones (tibia, femur, scapula) from Atp6ap2 cKO and control animals were dissected, and the bone marrow was flushed through an 100-μm nylon cell strainer (BD Biosciences) with PBS. BMDMs were cultured in DMEM containing 10 mM HEPES, 10 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% Glutamax, 10% (v/v) FCS, 5% horse serum, and 30% spent L929 culture supernatant at 9.5% CO₂, and cells were used in experimental setups after 7-10 days of differentiation. The murine macrophage-like cell line RAW264.7 (TIB-71) was from ATCC, and the murine macrophage-like cell line J774E (27) was kindly donated by Philip Stahl (Washington University, St. Louis, Missouri).

RNA Extraction, Reverse Transcription, and Quantitative Real-time-PCR

Total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel), and 0.2–1 μg of RNA was spent for reverse transcription using the RevertAid RT kit and random hexamer primers (Thermo Fisher Scientific). Specific assays for quantitative real-time PCR were created with the Universal Probe Library Assay Design Center, and PCR was performed in a Lightcycler 480 II (Roche Applied Science). Relative mRNA expression was calculated by normalizing Cₚ values to the logarithmic average Cₚ of the most stable house keeping genes (Tuba1a, Hprt1, and Sdha). The resulting DCₚ values were compared between genotypes for statistical analyses. Primer efficiency (E) was determined for each PCR by co-measurement of a set of serial cDNA dilutions to obtain E=1–ΔCp plots describing relative mRNA expression levels.

Western Blotting

Total cell lysates were generated by adding PBS containing 1% (v/v) Triton-X-100 and 1× Cmplete Protease Inhibitor Mixture (Roche Applied Science) to cells on ice for 20 min followed by sonication for 2 × 10 s using a Branson Sonifer 450 (level 7 in a cup horn, Emerson Industrial Automation) and centrifugation at 16,000 × g for 10 min at 4 °C. Protein concentrations of the resulting supernatants were measured with the Pierce BCA protein assay kit (Thermo Fisher Scientific), and samples were adjusted to 2 μg of protein/ml. 20 – 40 μg of protein were subjected to SDS-PAGE and analyzed by immunoblotting. Lysosomes were purified and immunoblotted as described (28) and used at 10 μg of protein per lane.

Subcellular Fractionation

Confluent RAW264.7 macrophage cultures were incubated for 2 h in the presence of 10 μM saliphenylhalamide A (SaliPhe) or DMSO and lysed in a Dounce homogenizer with 15 strokes in HB (8.6% (w/v) sucrose, 20 mM HEPES/KOH (pH 7.2), 0.5 mM EGTA) containing 1× Cmplete Protease Inhibitor Mixture (Roche Applied Science). Total lysates (fraction T) were separated into nuclei (fraction P1) and post nuclear supernatant (PNS, fraction S1) by a 15-min centrifugation step at 960 × g. For further fractionation, PNS were centrifuged at 128,000 × g for 60 min to yield fractions enriched in cytосolic (fraction S2) and membrane-bound (fraction P2) proteins. Pellets and supernatants were handled in equal volumes. Samples of 10 μl were taken from each fraction and subjected to Western blotting.

Co-immunoprecipitation

For co-immunoprecipitation analyses, RAW264.7 cells were grown to almost complete confluence. Where indicated, cells were preincubated for 2 h with 10 μM SaliPhe or DMSO as carrier control, and treatment was continued throughout the whole immunoprecipitation protocol. Cell lysates were generated using lysis buffer (40 mM HEPES (pH 7.4), 12.5 mM EDTA, 2.5 mM MgCl₂, 10 mM β-glycerophosphate, 10 mM NaF, 0.3% (w/v) CHAPS, 1× Cmplete Protease Inhibitor Mixture (Roche Applied Science)) and centrifugated at 16,000 × g and 4 °C for 10 min. Dynabeads Protein G (Life Technologies) were preincubated with rotation in 5% (v/v) BSA in lysis buffer for 2 h at 4 °C to block unspecific binding. 3 μg of soluble protein were pre-cleared of proteins with affinity to the bead matrix material by a 2-h incubation with 25 μl of Dynabeads Protein G at 4 °C, beads were removed, and the lysates were incubated in presence of primary antibody overnight at 4 °C with rotation. 50 μl of fresh, blocked Dynabeads were added to the lysate-antibody mix for 30 min at room temperature to capture immune complexes. Immunoprecipitated proteins bound to beads were then rinsed 3× in lysis buffer containing 150 mM NaCl. 50 μl of SDS-sample buffer was added per sample, and samples were heated for 5 min at 95 °C. 10 μl of each denatured immunoprecipitate were separated using 4–12% NuPAGE Novex Bis-Tris Protein Gels (Life Technologies) and immunoblotted. For detection of v-ATPase V₅ subunit B2, Clean Blot IP Detection Reagent (Life Technologies) was used instead of regular anti-rabbit horseradish peroxidase to avoid interference of denatured immunoprecipitation antibody fragments.

Immunofluorescence Analysis

Semi-confluent cultures of cells grown on coverslips were fixed in 4% (w/v) paraformaldehyde solution for 20 min at room temperature and permeabilized with 0.2% (w/v) saponin in PBS. Unspecific antibody binding was blocked by preincubating the fixed and permeabilized samples with 10% (v/v) FBS in 0.2% (w/v) saponin in PBS (blocking solution). Antibody staining was performed in blocking solution overnight at 4 °C (primary antibody) and for 1 h at room temperature (secondary antibody) in a humidified chamber. Coverslips were embedded in 17% (w/v) Mowiol 4–88 (Calbiochem), 33% (v/v) glycerol, 20 mg/ml DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma), and 5 μg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma) in PBS. An Olympus FV1000 confocal laser scanning microscope was used for image acquisition. Visualization of the endocytic pathway was achieved with dextran-Texas Red (M, 70,000, Life Technologies). Cells grown on coverslips were incubated with 0.5 mg/ml dextran-Texas Red in DMEM containing 1 mg/ml bovine serum albumin (BSA) for 30 min. After rinsing 3 times with PBS and incubation in DMEM + 1 mg/ml BSA for further 3 h, samples were processed for immunofluorescence analysis as described above. In a similar approach, the self-quenched fluorochrome DQ-BSA Red (Life Technologies) was used to assess proteolytic activities. Therefore, cells were cultured overnight in the presence of...
100 μg/ml DQ-BSA Red in DMEM containing 1 mg/ml BSA, rinsed 3 times with PBS, and processed for fluorescence labeling.

**Analysis of Lysosome pH**

Acidic cellular compartments were detected using the acido-tropic dye LysoTracker Red DND-99 (Life Technologies). Cells were incubated with 333 nM LysoTracker Red in DMEM only for 20 min at 37 °C, fixed in 4% (w/v) paraformaldehyde solution, and embedded or directly analyzed by live cell fluorescence microscopy.

Ratiometric measurement of lysosome pH was performed as described previously (29, 30). Briefly, endocytic compartments of the analyzed cells were loaded with dextran-Oregon Green 514 (0.5 mg/ml, 70,000 Mw, Life Technologies) overnight. To enable pH analysis of late endosomes and lysosomes only, early compartments were cleared of label by a 2-h chase in the absence of the dextran derivate. The addition of 200 nM bafilomycin A1 or 10 μg/ml nigericin for 15 min at the end of this chase period was used to collapse the lysosomal pH gradient. Ratiometric imaging was accomplished by exciting samples alternately at 440 or 488 nm. For an in situ calibration cells were sequentially incubated with K\(^{+}\)-rich buffer solutions (145 mM KCl, 10 mM glucose, 1 mM MgCl\(_2\), and 20 mM of either MES or acetate, pH 4.0 – 6.5) including 10 μM nigericin and then fixed at the end of each experiment. Lysosome pH was then interpolated from the generated calibration curve fitted to the Boltzmann equation.

**In Cellulo Latex Bead Phagocytosis Assays**

**Immunofluorescence Analysis**—Latex beads (1.1-μm diameter; Sigma) were opsonized with IgG (human or murine; Sigma) overnight at 4 °C to allow uptake by human FcyRII-expressing MEFs or murine macrophages. Cells were grown to semi-confluence on coverslips, and the media were replaced by serum-free DMEM at the point of latex bead administration. To synchronize uptake, beads were briefly spun down onto the cells at 300 x g for 1 min, and bead uptake was allowed for 15 min at 37 °C, 5% CO\(_2\). Latex bead excess was washed away with PBS, and fresh DMEM was added. Beads that have not been taken up were stained with anti-IgG antibodies coupled to Alexa Fluor 594 for 1 min, and the samples were processed for immunofluorescence staining of the marker proteins early endosome antigen 1 (EEA1) or LAMP-2, respectively. In the case of primary murine macrophages, co-staining with an anti-V\(_{o}\) a3 antibody was included to assess v-ATPase knockdown efficiency. Where indicated, 200 nM bafilomycin A1 or 10 μg/ml nigericin were used to alkalinize lysosomes during the 15-min incubation with latex beads and during the following chase periods.

A second approach to follow fusion between latex bead-containing phagosomes (LBPs) and lysosomes in cells was done in RAW264.7 cells. Briefly, endocytic compartments of RAW264.7 macrophages were loaded with 50 μg/ml BSA-rhodamine overnight. Cells were rinsed with PBS and incubated in the absence of BSA-rhodamine for 2 h to ensure labeling of late endosomes and lysosomes only. Treatment with 10 μM SaliPhe or DMSO, respectively, was started concurrently with the 2-h chase period and continued until cell lysis. Latex beads (1 μm diameter, Polysciences) opsonized with murine IgG Fc-fragments (Thermo Fisher Scientific) were then added in DMEM for 10 min. After washing away the excessive latex beads, RAW264.7 macrophages were incubated for a further 0 – 80 min. Cells were homogenized, and LBP were purified as in Becken et al. (28) but by placing the density gradient in a 2-mL minicentrifuge tube that was centrifuged for 30 min and 1250 × g at 4 °C in a swinging out table top centrifuge rotor. Ratiometric measurement of lysosome pH was performed as described previously (29, 30, 31).

**Electron Microscopic Analysis**—1 × 10\(^5\) bone marrow-derived macrophages were labeled with ferrous nanoparticles (10 nm) for 30 min followed by 3 h of incubation in fresh medium to ensure labeling of lysosomes only. 1-μm murine IgG-Fc fragment-coated latex beads were added for 10 min (multiplicity of infection = 13) followed by 3 rinses of the cells to remove non-ingested latex beads. Cells were incubated for 10 min or 120 min at 37 °C in fresh medium to allow phagosome maturation and then fixed in 2% (v/v) glutaraldehyde in 200 mM HEPES buffer. For epoxy resin embedding, cell pellets were washed with water, fixed with 2% (w/v) OsO\(_4\) (Electron Microscopy Sciences) containing 1.5% (w/v) potassium ferricyanide, and block-stained with 1.5% (w/v) uranyl acetate (Fluka) for 30 min. The cells were then dehydrated using a graded ethanol series and embedded in epoxy resin (Sigma). 70-nm-thick sections were cut on a Leica ultramicrotome Ultracut EM UCT (Leica Microsystems) using a diamond knife (Diatome) and stained with 0.2% (w/v) lead citrate (Taab) in 0.1 N NaOH for 10 s. Sections were analyzed with a CM100 transmission electron microscope (FEI). The images were recorded digitally with a Quemesa TEM CCD camera (Olympus Soft Imaging Solutions) and iTEM software v 5.1 (Olympus Soft Imaging Solutions).

The extent of the phagosome-lysosome fusion was categorized into arbitrarily defined classes of small, medium, and large volume transfer. Membrane deposits that contained <20 ferrofluid particles within a small and usually a pointed membrane profile protruding from the phagosome membrane were defined as “small.” “Medium” deposits were defined as containing >20 ferrofluid particles and an area of protrusion <½ the proportion of the latex bead. “Large” deposits of ferrofluid contained >20 ferrofluid particles, and the protrusions exceeded ½ of the latex bead area. The surface fraction of the phagosome membrane over small deposits of ferrofluid was analyzed from profiles of at least 20 phagosomes on isotropic sections per sample. Intersections of the phagosome membrane profile with the horizontal and vertical lines of a lined test grid were counted, and fractions were calculated from the total counts per sample.

**Analysis of In Vitro Fusion between Latex Bead-containing Phagosomes and Lysosomes**

Cell-free fusion of LBP with lysosomes was performed as previously described (28) with modifications. Lysosomes and LBP were isolated from RAW264.7 macrophages, and cytosol was prepared from RAW264.7. Where indicated, isolated organelles were preincubated for 10 min at 4 °C with 10 μM SaliPhe or DMSO before the addition of the remaining components of the
**v-ATPase in Membrane Fusion**

_in vitro_ fusion reaction (final concentrations: 1× ATP-regenerating system, 1× salt solution, 1 mM dithiothreitol, 2 mg/ml RAW264.7 cytosol). 10 μM SaliPhe or DMSO were present during the whole fusion reaction. After 30 min at 37 °C, the samples were set on ice for 5 min and incubated with 0.2 mg/ml proteinase K (Qiagen) for 15 min. Then 1.75 mg/ml phenylmethylsulfonyl fluoride was added and the volume increased to 200 μl with HB. Latex bead phagosomes were isolated from the samples, placing them on top of a 1-ml HB/25% (w/v) sucrose cushion in a swing-out rotor (1800 × g, 30 min). The collected phagosomes were diluted in HB and added to coverslips in 24-well plates followed by centrifugation (690 × g, 15 min, 4 °C) and fixation (3% (v/v) formaldehyde, 2.5% (v/v) glutaraldehyde in HB) overnight. Remaining aldehyde was quenched with 0.1 M salicylhydroxamic acid (Escherichia coli DH5α or Listeria innocua serovar 6a (NCTC 11288) in serum-free DMEM for 15 min at 37 °C. Bone marrow-derived macrophages were infected at a multiplicity of infection of 5 with Escherichia coli DH5α or Listeria innocua serovar 6a (NCTC 11288) in serum-free DMEM for 15 min at 37 °C. After rinsing cells 3 times with PBS to remove external bacteria, DMEM containing 10 μg/ml gentamicin (Roth) was added to kill the remaining extracellular bacteria. Macrophages were lysed at 0, 20, or 60 min after infection with 0.1% (v/v) Triton X-100, and serial dilutions were plated on nutrient agar plates to quantify colony forming units the next day. Numbers of colony forming units at 0 min were set as 100%.

**Results**

_V₀ Subunit a3 Deficiency Despite Normal pH of the Lysosome—_ In a genetic-based approach to analyze the role of the v-ATPase subunit a3 in membrane fusion, we used cells from _Tcirg1<sup>−/−</sup>_/ _oc_/ _oc/oc_ mice, which are homozygous for the osteosclerosis (oc/oc) mutation and naturally lack the transmembrane a3-subunit of the v-ATPase (24). This subunit is particularly relevant here because in yeast the a-subunit has been previously implicated in the terminal phase of membrane fusion (11, 12) and the a3 isoform is the relevant a-isoform in macrophage phagosomes and lysosomes (32). The a3-deficient _oc/oc_ mice suffer from severe osteopetrosis and die early (24, 33) so that the preferred cell type, primary macrophages, could not be obtained. However, we succeeded in generating fibroblast cell lines from these mice, and we confirmed their lack of subunit a3-expression by Western blotting (Fig. 1A), quantitative real-time PCR (Fig. 1B), and immunostaining (Fig. 1C). The mRNA and protein concentration of the subunit a1 was increased in the a3-deficient cells, whereas the subunit a2 shows unaltered expression level. Subunit a4 was only expressed at a low rate when compared with the other three isoforms in wild-type cells, and a4-mRNA concentration was not affected upon deletion of subunit a3 (Fig. 1, A and B). Concentrations of the _V₀_ subunits d1 and c as well as the _V₁_ subunit B2 remained unchanged (Fig. 1A). Surprisingly, fluororatiometric pH determination using Oregon Green 514 demonstrated no difference in lysosome pH in a3-deficient versus wild-type cells (Fig. 1, D and E), and acidification, visualized with Lyso-Tracker Red staining, was highly sensitive to bafilomycin A1 treatment (Fig. 1, F and G) and, hence, dependent on a functional v-ATPase complex.

Phagosome-Lysosome Fusion Occurs in the Absence of _V₀_ Subunit a3—_MEFs are non-professional phagocyte fibroblast cells. To study phagosome maturation, wild-type and _oc/oc_ MEFs were stably transfected with an Myc-tagged CD32-cDNA coding for the human IgG receptor FcyRII (Fig. 2A), which mediates phagocytosis of IgG-coated particles. Cells were incubated for 15 min with IgG-coated latex beads, and maturation of the LBP was monitored over time by determining the colocalization of the internalized beads with the EEA1 and with late endosomal/lysosomal LAMP-2 (Fig. 2B). Immediately after uptake, most beads colocalized with the early endosome marker EEA1 in both genotypes (Fig. 2, B and C). Co-localization of the lysosome membrane protein LAMP-2 with LBP increased steadily and was observed with almost all phagosomes at 60 min of chase. Kinetics of EEA1 loss from, and LAMP-2 acquisition to some membrane protein LAMP-2 with LBP increased steadily (Fig. 1, D and E). The mRNA and protein concentration of the _V₀_ subunits d1 and c as well as the _V₁_ subunit B2 remained unchanged (Fig. 1A). Surprisingly, fluororatiometric pH determination using Oregon Green 514 demonstrated no difference in lysosome pH in a3-deficient versus wild-type cells (Fig. 1, D and E), and acidification, visualized with Lyso-Tracker Red staining, was highly sensitive to bafilomycin A1 treatment (Fig. 1, F and G) and, hence, dependent on a functional v-ATPase complex.

**Statistical Analysis**

All values are expressed as the mean ± S.D./S.E. and analyzed via two-tailed, unpaired Student’s t tests or one-way analysis of variance followed by a Tukey-Kramer test using GraphPad Instat 3 software (*, p < 0.05; **, p < 0.01).
suggested a mechanism of phagosome maturation that is independent of luminal pH.

It is also of note that dextran (Fig. 3A) and transferrin (data not shown) were taken up to the same extent by both wild-type and mutant cells. Moreover, lysosome hydrolase activities (Fig. 3B) and processing of cathepsin-D and cathepsin-L (Fig. 3C) also did not differ between a3-deficient and wild-type MEF cells in steady state. These experiments indicate that fusion events within the endocytic pathway are not affected by the lack of the a3 subunit.

Because we could not exclude possible compensatory processes for a3 by the subunit a1, which might mask acidification-independent functions, we further studied the consequences of a deletion of all isoforms in professional phagocytes, such as macrophages.

Disruption of v-ATPase Assembly after Conditional Knockout of Atp6ap2—We took advantage of a recently generated cKO mouse line in the gene for ATP6AP2. The encoded protein is an accessory v-ATPase subunit and is also known as prorenin receptor. This protein is a central factor in the assembly of the v-ATPase, and its removal results in the instability and disappearance of complete V0 complexes (21). The generated Atp6ap2<sup>−/−</sup> MEFs were treated for 1 week with polyinosinic-polycytidylic acid to induce Cre expression in interferon-α-responsive cells that include peritoneal macrophages and stem cells that we used to induce BMDM.
phages were analyzed 10–17 days after induction and differen-
tiation to BMDMs. This revealed that a deficiency of ATP6AP2
could indeed be obtained and that the concentrations of several
V0 subunits, including subunit a isoforms 1–3, were signifi-
cantly reduced in the cKO macrophages (Fig. 4A). However,
loss of ATP6AP2 did not affect mRNA expression of these and
additionally tested subunits (Fig. 4B), suggesting posttranscrip-
tional events leading to the loss of V0 subunits. Subunit a3
showed the highest mRNA expression level of the four a-iso-
forms, whereas a3 protein was almost completely missing, as
demonstrated by immunoblotting and immunofluorescence
microscopy of differentiated macrophages (Fig. 4, A and C).
Therefore, Atp6ap2 cKO is a suitable model to analyze an
almost complete absence (up to 96%) of V0 subunits of the
v-ATPase.

In these cells the lysosome pH was only slightly less acidic
than in wild-type cells (Fig. 4, D–F), enabling us to study an
pH-independent effect on lysosome fusion with phagosomes. It
is interesting to note that when treating cells with bafilomycin
A1, ATP6AP2-deficient macrophages were somewhat more
susceptible to the drug than control cells (Fig. 4E), probably also
reflecting the reduced number of v-ATPase complexes in the
Atp6ap2 knock-out macrophages. As a measure of the biolog-
ical relevance of v-ATPase in phagosome maturation, the kill-
ing capacities toward bacteria of BMDMs from wild-type and
induced Atp6ap2 knock-out mice were compared (Fig. 4G).

**FIGURE 2.** Loss of V0 subunit a3 does not influence phagosome-lysosome fusion. A, total cell lysates of wild-type and a3-deficient MEFs, stably transfected with human FcγRII-myc, were probed with the indicated antibodies in Western blot analysis. GAPDH was used to control for equal protein load. B, analysis of latex bead phagocytosis in FcγRII-myc expressing MEFs. Cells were pulsed with human IgG-opsonized latex beads for 15 min, and maturation of latex bead-containing phagosomes was monitored for chase periods from 0 to 60 min. External latex beads were labeled with an anti-IgG antibody, and samples were fixed for immunostaining. Colocalization rates between internalized latex beads (no hIgG-signal) and EEA1 or LAMP-2 were determined by laser-scanning confocal microscopy after the indicated chase periods. C, representative fluorescence images are shown for experiments conducted in B including magnified regions of interest. Arrows point to areas with colocalization (scale bars = 10 μm). DIC, differential interference contrast. hIgG, human IgG. D, influence of manipulation of luminal pH on phagosome maturation. Experiments in B were conducted in the presence of the v-ATPase inhibitor bafilomycin A1 (200 nM), the ionophore nigericin (10 μg/ml), or carrier control (DMSO) with the treatment starting either 15 min before (bafilomycin A1, DMSO) or concurrent with latex beads incubation (nigericin). Percentages of phagosomes colocalizing with LAMP-2 and EEA-1 were determined by laser-scanning confocal microscopy. A minimum of 100 phagosomes was analyzed per condition. Data are presented as the means ± S.E. from 2–3 independent experiments.
FIGURE 3. Endocytic maturation and degradation in lysosomes is normal in a3- and ATP6-deficient cells. Lysosome functions were analyzed in wild-type and a3−/− MEFs and wild-type and Atp6ap2 cKO macrophages. A, visualization of vesicle maturation upon macropinocytosis and degradation within lysosomes. Cells were incubated either in the presence of dextran-Texas Red (Dex-TR; 0.5 mg/ml) for 30 min plus a 2-h chase period or overnight in the presence of DQ-BSA (0.1 mg/ml). Samples were fixed and immunolabeled with anti-V0 a3 and anti-LAMP-2 antibodies. Distribution of the fluorescent marker proteins and their signal intensities were similar in the analyzed genotypes of both cell types. Shown are representative immunofluorescence images (scale bars = 10 μm).

B, total β-hexosaminidase activity was comparable between a3-deficient and wild-type MEFs and between Atp6ap2 cKO and wild-type macrophages, respectively. Shown are the means ± S.E. from 7–9 independent experiments.

C, intracellular proteolytic processing of cathepsins D and L was analyzed by Western blotting in the cell types mentioned in A and B. No impairment of enzyme maturation is visible. Arrows point to proform (P), intermediate (I), and mature (M) forms of the respective cathepsin. Lysates from cathepsin D (CtsD−/−) and cathepsin L (CtsL−/−) knock-out MEFs were used to control for antigen specificity of the antibodies (*, unspecific signal).
**v-ATPase in Membrane Fusion**

![Diagram](image)

**FIGURE 4. Killing of bacteria within macrophages is increased by a reduced v-ATPase concentration.** Conditional knock-out of the v-ATPase accessory protein 2 (ATP6AP2) was induced in Atp6ap2<sup>flx/flx</sup>Mx1-Cre<sup>+/−</sup> mice by polyinosinic-polycytidylic acid treatment, and mice were sacrificed 10 days post induction for isolation of peritoneal (A–F) or generation of BMDMs (G). Cells derived from Atp6ap2<sup>flx/flx</sup>Mx1-Cre<sup>+/−</sup> mice served as wild-type controls. A, Western blot analysis of v-ATPase expression. Cells were lysed and subjected to immunoblotting against v-ATPase subunits. Anti-ATP6AP2 antibodies were applied to determine knock-out efficiency. B, mRNA expression levels of different v-ATPase subunits as determined by quantitative real-time-PCR. Values are presented in relation to the most stable housekeeping genes as the means ± S.E. from three (knock-out) to four (wild-type) macrophage preparations. *, p < 0.05 according to unpaired two-tailed Student’s t test. C, immunofluorescence analysis of localization of V<sub>0</sub> subunit a3. Macrophages were fixed and labeled with anti-V<sub>0</sub>a3 and anti-LAMP-2 antibodies. Colocalization between a3 and LAMP-2 was measured to be 72% (Pearson’s correlation coefficient = 0.78) in wild type and 24% (Pearson’s correlation coefficient = 0.27) in Atp6ap2 knock-out cells. D and E, primary macrophages were analyzed for lysosome acidification by LysoTracker Red staining. D, representative fluorescence images of macrophages either untreated or pretreated with bafilomycin A1 (100 nM) for 15 min. E, a dose-response curve was generated by measuring LysoTracker Red intensity after 15 min of pretreatment with various concentrations of bafilomycin A1 (0–200 nM). Shown are mean intensities in relation to DMSO-treated samples ± S.D. from two independent experiments. F, lysosome pH in Atp6ap2 cKO cells was determined using ratiofluorometric dextran-Oregon Green S14 measurements. Treatment for 1 h with bafilomycin A1 (200 nM) yielded a pH of >6.5 (not shown). Data are presented as the means ± S.E. from four (wild-type) to six (knock-out) macrophage preparations. *, p < 0.05 according to unpaired two-tailed Student’s t test. G, killing of bacteria within BMDMs. Macrophages were infected with either *E. coli* or *L. innocua* (multiplicity of infection = 5) (both non-pathogenic bacteria), and colony forming units recovered from agar plates relative to the numbers recovered from 0-time samples. Shown are the means ± S.E. from 2–5 independent experiments. *, p < 0.05 according to unpaired two-tailed Student’s t test between genotypes. Scale bars = 10 μm; insets show differential interference contrast images.

Surprisingly, ATP6AP2-depleted macrophages killed *E. coli* (Gram-negative, avirulent) and *L. innocua* (Gram-positive, avirulent) rather more efficiently, suggesting that the absence of the most of v-ATPase complexes did not affect their microbialic functions. Compromising lysosome pH by application of bafilomycin A1 also did not change the killing capacity of macrophages toward *E. coli* (data not shown), implying bactericidal mechanisms, which are independent on an acidic milieu. Similar to a3-deficient MEF cells, endocytic uptake and delivery to lysosomes of dextran and BSA (Fig. 3A) also were not affected in Atp6ap2 cKO macrophages; neither were maturaion and activity of lysosomal hydrolases (Fig. 3, B and C).

**Phagosome-Lysosome Fusion in ATP6AP2-depleted Macrophages**—Knock-out of Atp6ap2 exclusively in cells of the myeloid lineage, including macrophages, due to *LysM*-mediated Cre deletion similarly resulted in the disappearance of the a<sub>1</sub>- and a<sub>3</sub>-V<sub>0</sub>a subunits. Nevertheless in these cells we found unaltered lysosome acidification and a significant colocalization of LAMP-2 with phagocytosed latex beads (Fig. 5), suggestive of unaltered phagosome maturation. To analyze at an ultrastructural level whether phagosome maturation was affected, we performed transmission electron microscopy experiments. The endocytic pathway of wild-type and induced Atp6ap2 cKO BMDMs was prelabeled with 10-nm ferroparticles and then
incubated with 1.1 μm latex beads for 10 min followed by removal of external beads and further incubation for 10 or 120 min (Fig. 6A). Transmission electron microscopy visualization of phagosomes (Fig. 6B) revealed three types of nanoparticle-containing fusion profiles between phagosomes and lysosomes that were categorized as small, medium, and large (Fig. 6C). A significantly higher number of tight-fitting (small) membrane profiles per bead was observed in Atp6ap2 cKO cells compared with wild-type cells. Medium and large profiles were observed at a much lower frequency in both genotypes with an even more reduced number in the Atp6ap2 knock-out cells compared with wild-type cells. Medium and large profiles were observed with murine IgG (mlgG)-opsonized latex beads for 15 min and incubated for a further 60 min after washout of bead excess. Before fixation of the cells for immunofluorescence analysis, external latex beads were labeled with an anti-mlgG antibody (1 min). Lysosomal structures are visualized with an anti-LAMP-2 antibody. An anti-V0a3 staining was performed to control for Atp6ap2 knock-out. Scale bars = 10 μm. DIC, differential interference contrast.

FIGURE 5. Knock-out of Atp6ap2 in LysM-Cre transgenic mice. Peritoneal macrophages harvested from Atp6ap2<sup>Fl<sup>ox</sup>/Fl<sup>ox</sup></sup> LysM-Cre<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> (control) mice were analyzed for v-ATPase expression and activity. A, expression of Atp6AP2 full length (fl) and C-terminal fragment (CTF) as well as expression of designated v-ATPase subunits was analyzed by immunoblotting of cell lysates from macrophages. β-Actin was used to control for equal protein load. B, quantification of lysosome pH with ratiometric dextran-Oregon Green 514 measurements. Data are presented as the means ± S.E. from five macrophage preparations for each genotype. C, uptake and intracellular delivery of latex beads. Atp6ap2 LysM-KO and control cells were incubated with murine IgG (mlgG)-opsonized latex beads for 15 min and incubated for a further 60 min after washout of bead excess. Before fixation of the cells for immunofluorescence analysis, external latex beads were labeled with an anti-mlgG antibody (1 min). Lysosomal structures are visualized with an anti-LAMP-2 antibody. An anti-V0a3 staining was performed to control for Atp6ap2 knock-out. Scale bars = 10 μm. DIC, differential interference contrast.

Discussion

Does proton-pumping v-ATPase regulate and possibly even catalyze phagosome-endosome and phagosome-lysosome fusion, and how does it participate in pathogen killing? Recent work by other groups suggested that the V0 sector, the membrane integral parts of v-ATPases, acts downstream of SNARE complex formation and contributes to lipid mixing and membrane fusion (11, 35, 36). V0 sectors in only one of the two fusion partners were reported to be sufficient to support fusion (35). The fusion of intermembrane fusion complexes appeared to be not a prerequisite for phagosome maturation. Recently, saliphenylhalamide A, a v-ATPase inhibitor, was described to lock V0 and V1 sectors of v-ATPases together (34). Because it has been postulated (11, 35) that the V0 subunit catalyzes membrane fusion only when being free of V1, we investigated the V0-V1 locking effect <i>in vitro</i>. The advantage of this drug is that both the reduced availability of free V0 subunit and the effect of increased lysosome pH can be studied in one sample. We observed a clearly increased amount of assembled complexes in the presence of the equipotent salicylhalamide analog saliphenylhalamide A (Fig. 8, A–C) as well as loss of LysoTracker Red signals caused by an increased lysosome pH (Fig. 8D). Yet, there was no effect on phagosome-lysosome fusion <i>in vitro</i> (Fig. 8E). Saliphenylhalamide A also did not change the degree of fusion of BSA-rhodamine-labeled lysosomes with latex bead-containing phagosomes in macrophages (Fig. 8F), again questioning a central role of v-ATPase participation in fusion.
require calcium release but was independent of the proton pumping activity of the v-ATPase (12, 37). We have chosen pharmacological and genetic approaches to analyze the contribution of the v-ATPase complex to fusion between phagosomes and endo(lyso)somes, which contain large quantities of v-ATPase. In summary, surprisingly, neither of the above v-ATPase manipulations yielded a significant change in the endocytic or phagocytic uptake, in lysosome acidification, or in phagosome-lysosome fusion. Our data suggest that neither v-ATPase in general nor the a3 subunit in particular have an apparent direct role as a promoter of phagosome-lysosome fusion in our systems.

The role of the v-ATPase complex and specifically the membrane-bound V0 sector as a fusion-enhancing factor has been a matter of extensive investigations (13, 38). With respect to the phagocytic pathway, this role has only been studied by looking at apoptotic body removal in zebrafish brain (16) where the a1-subunit of v-ATPase, despite an unaltered lysosome pH, was
critical for fusion. This suggests that different phagocytic cargos with different degrees of danger signals, e.g. apoptotic blebs versus IgG-opsonized latex bead phagosomes or bacteria, may feed into different maturation pathways. Also, the use of different organisms and tissues, zebrafish brain versus mouse macrophage, and of different strategies for gene inactivation, morpholino addition versus conditional knock-out, may have caused this. Another suggested correlation between v-ATPase possession and phagosome maturation comes from phagosomes containing Mycobacterium tuberculosis. These phagosomes almost completely lack v-ATPase, and they are arrested at an early stage along the endocytic/phagocytic continuum (39). This correlation suggested that the lack of the proton pump may not only be indicative for altered phagosome trafficking of this pathogen-containing phagosome but may actually cause it.

Our approach to study proton-pumping-independent contributions of v-ATPases to membrane fusion was the generation of cell lines disturbed in v-ATPase complex assembly that contain fewer V₀ complexes (62–96% reduction varying between different subunits), likely due to increased protein degradation. Furthermore, we have used cells with a knock-out of the a3 subunit. Both types of experiments challenged the cellular functions of the transmembrane V₀ sector (7). Of the four possible isoforms of the v-ATPase a-subunit, a3 is arguably the most important one on macrophage lysosomes and may be relevant for phagosome acidification (32). It was suggested that the a3 subunit is likely the most prominent in J774 and RAW264.7 macrophage cell lines (40, 41) in which it is localized to phagosomes and needed for bactericidal function (32). a3-knock-out mouse embryonic fibroblasts have normal endocytic functions and unaltered activities of lysosomal hydrolases at a normal lysosome pH. The complete lack of a3 also did not change the kinetics of phagosome maturation, strongly suggesting that fusion of late endosomes/lysosomes with phagosomes occurs to the same extent in the absence of this a-subunit of the v-ATPase. It may well be that at least a portion of the a3 subunit is replaced by the a1-subunit of v-ATPase as suggested by the increased expression of this subunit in the a3-deficient MEF cells. Expression levels of other subunit a-isoforms were unaltered or very low in protein and mRNA levels, making a compensatory role of these subunits unlikely. A limited functional compensation for subunit a3 by subunit a1 has been discussed in osteoclasts, a cell population with predominant expression of subunit a3 and only low levels of subunit a1 (42). Knock-out of a3 did not suffice to completely block the osteoclast’s v-ATPase function (43).

Our studies using bone marrow-derived macrophages from mice deleted in Atp6ap2, a gene essential for vacuolar ATPase assembly, revealed that deletion resulted in a significant drop in the concentrations of all v-ATPase V₀ subunits. Similar to the situation in cardiomyocytes and kidney (21, 44, 45), the presence of ATP6AP2 appears to be a prerequisite for the v-ATPase complex in the endoplasmic reticulum. Taking the pivotal role of the v-ATPase complex into account, it is not surprising that a complete deficiency of v-ATPase may lead to early cell death (46). We expected that the pronounced loss of the v-ATPase levels would cause both a strong reduction in lysosome acidification and in membrane fusion between late endocytic organelles. Surprisingly, the ability to acidify these compartments was only slightly, if at all, affected in the absence of functional v-ATPase. This can possibly be explained by the presence of some v-ATPase holo-complexes, which may also be a prerequisite for the survival of the knockdown cells. The expression levels of all a-isoforms, except for the isofrom a4, which is not expressed in macrophages, were significantly lower in Atp6ap2 knock-out than in wild-type macrophages. It is likely that the remaining v-ATPase complexes are sufficient to fulfill their proton-pumping function, as acidification was still bafilomycin A1-sensitive. However, less bafilomycin A1 is needed to increase the pH in Atp6ap2 knock-out cells, suggesting a lower number of functional v-ATPase complexes.

The presence of a normally low lysosome pH allowed us to monitor the fusion capacity of lysosomes with phagosomes without the need to consider abnormal acidification as a reason for fusion failure.
for any of the phenotypes we would observe. After all, distur-
bances of trafficking events in the endocytic pathway caused by
pH neutralization have been described (5). Despite the signifi-
cant drop in the number of v-ATPase complexes in Atp6ap2
knock-out cells, all tested functions of endocytic and phago-
cytic compartments were unaffected, and phagosome-lyso-
some fusion as well as killing of bacteria was even somewhat
pronounced. We expected that the large decrease in complex
concentration would affect the number of fusion events. How-
ever, no effect on fusion was observed. Hence, the residual lev-
els of v-ATPase complexes may still contribute to membrane
fusion.

**FIGURE 8. Increased formation of V0/V1 complexes does not affect phagosome-lysosome fusion.** Influence of the v-ATPase inhibitor SaliPhe on in vitro and in cellulo fusion between phagosomes and lysosomes was analyzed in RAW264.7 macrophages. A–C, effect of SaliPhe on v-ATPase complex formation. A, subcellular fractionation of RAW264.7 cells after 2 h of treatment with 10 μM SaliPhe or DMSO as indicated. T, total lysate; S1, low speed supernatant/perinuclear supernatant; P1, low speed pellet/cells and nuclei; S2, high speed supernatant/cytosolic proteins; P2, high speed pellet/membrane bound proteins. SaliPhe treatment shifts v-ATPase V1 subunits B und E1 from the cytosolic to the membrane-bound fraction. LAMP-1 and V0C localization were not influenced by SaliPhe. Shown are representative immunoblots from two independent experiments (*, unspecific signal). B, SaliPhe increases V0/V1 interaction. Immunoprecipitates (anti-V1B2) were prepared from cells treated as in A and analyzed for co-precipitation of V0 subunits. LAMP-1 was included as the negative control for interaction. Representative blots from two independent experiments are shown. IP, immunoprecipitation. C, lysosomes from RAW264.7 cells were co-labeled with BSA-rhodamine and nanomagnets and segregated via magnetic sorting. Isolated organelles were then incubated for 60 min under in vitro fusion conditions and processed for Western blotting. Where denoted, isolated lysosomes were pretreated for 10 min with SaliPhe (10 μM), and the treatment was continued throughout the experiment. Staining with the indicated antibodies shows more V1 bound to lysosomes when SaliPhe was present during the incubation. D, lysosome pH is increased by SaliPhe treatment. RAW264.7 cells were treated with 200 nM bafilomycin A1 or 10 μM SaliPhe for 100 min before LysoTracker Red staining. Shown are representative fluorescence images (insets: differential interference contrast channel). Scale bar = 10 μm. E, in vitro fusion experiments were carried out with lysosomes treated as described in C, and LBPs were isolated from macrophages. Both organelle types were either pretreated for 10 min with SaliPhe or left untreated. In vitro fusion was then allowed to proceed for 60 min at 37 °C in the presence or absence of SaliPhe or carrier (DMSO). Incubation at 4 °C served as a negative control. F, influence of SaliPhe on in cellulo fusion of BSA-rhodamine-labeled lysosomes with LBPs. RAW264.7 macrophages were cultivated in the presence of BSA-rhodamine overnight, and the label was chased into lysosomes for 2 h while incubating with SaliPhe or DMSO. Macrophages were then allowed to ingest latex beads for 10 min and incubated for a further 0, 20, or 80 min with the addition of SaliPhe/DMSO. Cells were lysed, and phagosomes were isolated to analyze the frequency of colocalization between BSA-rhodamine and LBPs. Data are presented as the means ± S.E. from three independent experiments.
The removal of the bulky V_o transmembrane protein complex (47, 48) from membranes might decrease steric hindrance for membrane fusion catalyzed by other factors such as the SNARE complexes, thereby increasing fusion rates. In this context it is interesting to note that the size of protrusion of the yeast V_o/V_1 complex from the membrane has been estimated to be 415 nm (49). The assumption that this huge complex might impair the contact between membranes also finds support in our electron microscopy-based observation of more fusion events in the Atg6ap2-deleted macrophages as compared with the wild-type cells.

This interpretation is in contrast to studies in yeast and mammalian cells that implicated a direct V_o sector involvement in fusion that is independent of the intraluminal pH (11–13, 16, 37). However, some of this work is disputed, as a recent study on homotypic vacuole fusion in yeast argues strongly that acidification rather than the presence of the v-ATPase is a major driving factor of membrane fusion (17).

Contrary to these findings, in our experiments, acidification also did not seem to be a driving force, as neither nigericin nor saliphenylhalamide A did not affect the extent of phagosome-lysosome fusion under conditions of increased lyso-some pH. Also, locking many of the V_0-V_1 sectors together by heat-killed bacteria. These observations suggest efficient phagosome-lysosome fusion under conditions of increased lysosome pH. Also, locking many of the V_o-V_1 sectors together by saliphenylhalamide A did not affect the extent of phagosome-lysosome fusion, although it could have been expected that this locking would reduce the supply of free V_o and place extra bulky protein complexes between the two membranes destined to fuse (35).

Taken together, our data with cells either mostly lacking the v-ATPase complex or completely lacking its a3 subunit did not reveal an absolute necessity of the v-ATPase in fusion. Strongly supportive of these findings is the fact that reconstituted fusion with purified components of yeast vacuoles proceeded in the complete absence of a v-ATPase complex (50). The same was true for reconstituted homotypic early endosome fusion (51).

Another piece of data pointing to a non-mandatory role for v-ATPases in membrane fusion is that compartments that naturally lack a v-ATPase complex, such as postmitotic nuclear vesicles (52), can still fuse. Although the above arguments and data do not unequivocally exclude a regulatory role of v-ATPases in fusion (53), they argue that the presence of the v-ATPase is not obligatory and that acidification-independent roles of v-ATPases in fusion may rather be the exception than the rule.

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