Host Resistance to Intracellular Infection: Mutation of Natural Resistance-associated Macrophage Protein 1 (Nramp1) Impairs Phagosomal Acidification

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Summary

The mechanisms underlying the survival of intracellular parasites such as mycobacteria in host macrophages remain poorly understood. In mice, mutations at the Nramp1 gene (for natural resistance-associated macrophage protein), cause susceptibility to mycobacterial infections. Nramp1 encodes an integral membrane protein that is recruited to the phagosome membrane in infected macrophages. In this study, we used microfluorescence ratio imaging of macrophages from wild-type and Nramp1 mutant mice to analyze the effect of loss of Nramp1 function on the properties of phagosomes containing inert particles or live mycobacteria. The pH of phagosomes containing live Mycobacterium bovis was significantly more acidic in Nramp1-expressing macrophages than in mutant cells (pH 6.6 ± 0.05 versus pH 5.5 ± 0.06, respectively; P < 0.005). The enhanced acidification could not be accounted for by differences in proton consumption during dismutation of superoxide, phagosomal buffering power, counterion conductance, or in the rate of proton “leak”, as these were found to be comparable in wild-type and Nramp1-deficient macrophages. Rather, after ingestion of live mycobacteria, Nramp1-expressing cells exhibited increased concanamycin-sensitive H+ pumping across the phagosomal membrane. This was associated with an enhanced ability of phagosomes to fuse with vacuolar-type ATPase-containing late endosomes and/or lysosomes. This effect was restricted to live M. bovis and was not seen in phagosomes containing dead M. bovis or latex beads. These data support the notion that Nramp1 affects intracellular mycobacterial replication by modulating phagosomal pH, suggesting that Nramp1 plays a central role in this process.

Key words: mycobacterium tuberculosis • phagosome • phagocytosis • macrophage • proton pump
Innate resistance or susceptibility to mycobacterial infections has been detected in human populations (14, 15) and in animal models such as the laboratory mouse (16, 17). In a few instances, the genetic determinants of resistance or susceptibility have been identified. In the mouse, the Bcg/lty/Lsh locus on chromosome 1 confers natural resistance to infection with a group of seemingly unrelated intracellular parasites including several mycobacterial species (M. bovis, M. leprae, M. smegmatis, M. avium, and M. intracellulare), Salmonella typhimurium, and Leishmania donovani (17–22). Susceptibility in vivo is expressed as an uncontrolled microbial replication in the spleen and liver early during infection, which is caused by an inability of the tissue macrophages to restrict intracellular proliferation (20, 22–25). The Bcg locus has been identified by positional cloning (N ramp1 [natural resistance–associated macrophage protein]; reference 20), and has been shown to encode an integral membrane phosphoglycoprotein of 110 kDa that is expressed almost exclusively in macrophages (26). Inbred strains of mice, susceptibility to infection is associated with a single amino acid substitution in N ramp1 (G169R), which causes rapid degradation of the protein (21). In vivo typing of animals showing either a loss of function null allele (knockout) or a gain of function N ramp1 transgene (N ramp16169 in C57BL/6J mice) have established that N ramp1 and Bcg are indeed allelic (27, 28). In humans, N ramp1 mRNA is expressed in both granulocytes and mononuclear phagocytes, and polymorphic variants at or near N ramp1 have been found associated with increased susceptibility to leprosy (29). The pleiotropic effect of mutations at N ramp1 on resistance to infections with antigenically unrelated microbes suggests that this protein plays a key role in basic antimicrobial defense mechanisms of phagocytes. Immunolocalization studies have shown that N ramp1 is expressed in the late endosomal/early lysosomal (lysosomial–associated membrane protein [LAMP] 1–positive) compartment of the macrophage, and is recruited to the membrane of the phagosome through fusion events during the maturation process that follows phagocytosis (30). Therefore, N ramp1 is likely to confer resistance to mycobacterial infection by directly altering the phagosomal milieu.

The generation of an acidic interior is generally believed to be essential to the microbicidal activity of phagosomes (9, 31). Acidification of the phagosomal lumen, which is initiated and maintained primarily by the action of vacuolar–type proton ATPases (V-ATPases; references 9, 32), can exert a direct toxic effect on internalized bacteria. In addition, it is required for the activation of some lysosomal hydrolases, which typically have low pH optima (33, 34). Importantly, phagosomes containing mycobacteria fail to acidify normally, at least in part because of exclusion of V-ATPases (9, 11, 35), caused by alterations in fusogenic properties of the mycobacterial phagosomes.

These results together with the established phagosomal location of N ramp1 prompted us to investigate the possibility that N ramp1 may control mycobacterial replication through effects on phagosomal maturation and acidification. To this end, we compared the properties of phagosomes induced by M. bovis in macrophages from either normal, N ramp1 positive mice or from animals bearing a null mutation at the N ramp1 locus (27, 28). The results indicate that N ramp1 plays a key role in the events leading to phagosomal acidification, and ultimately, inhibition of mycobacterial replication.

Materials and Methods

Materials, Solutions, and Antibodies. Nigericin, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) acetoxymethyl ester, O regen green, N -hydroxysuccinimidyl 5-(and 6-)carboxyfluorescein (NHS-CF), FITC–conjugated dextran, FITC–conjugated human holotransferrin, and Texas red–conjugated dextran were from Molecular Probes, Inc. (Eugene, OR). Streptolysin O (SLO) was obtained from Dr. S. Bhakdi (Institute for Medical Microbiology, Johannes Gutenberg University, Mainz, Germany). Concanamycin was obtained from Kamyla Biochemical Company (Thousand Oaks, CA). Diphenylene iodonium was synthesized in our laboratory as described (36). Valinomycin was from Calbiochem-Novabiochem Corp. (La Jolla, CA). All other chemicals were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO), Fisher Scientific Co. (Pittsburgh, PA), or Pharmacia Biotech (Piscataway, NJ).

Polyclonal antibodies to the 39-kD subunit of the V-ATPase were raised by injecting rabbits with a glutathione-S-transferase fusion protein encoding the entire subunit and were then affinity purified as described (9). mAbs to LAMP-2 were obtained from the Developmental Studies Hybridoma Bank, maintained by both the University of Iowa (Ames, Iowa) and Johns Hopkins University School of Medicine (Baltimore, MD; reference 37). Cy3–conjugated donkey anti–mouse, anti–rabbit, and anti–rat IgG and FITC–conjugated donkey anti–rat IgG were obtained from Jackson ImmunoResearch Labs (West Grove, PA).

PBS consisted of (mM): 140 NaCl, 10 KCl, 8 sodium phosphate, 2 potassium phosphate, pH 7.4. Powdered medium RPMI 1640 (H CO3 ––free; Hepes buffered) was from Sigma Chemical Co. The K +–rich medium contained (mM): 140 KCl, 5 glucose, and 15 Hepes, pH 7.4. In all cases the osmolarity was adjusted to 290 ± 5 mosM with the major salt. Permeabilization buffer consisted of (mM): 140 potassium glutamate, 5 NaCl, 10 glucose, 10 Hepes K, and 0.1 EGTA, pH 7.35.

Cell and Bacterial culture. Mice expressing wild-type N ramp1 (strain 129/sv) were obtained from Taconic Farms (Germantown, NY). Knockout mice with a null mutation at the N ramp1 locus were generated on a 129/sv genetic background, as described (27, 28). Resident peritoneal macrophages were obtained from N ramp1 mutant (N ramp1 knockout) and wild-type mice as described (38). In brief, the peritoneal cavities of 6–8–wk-old mice were lavaged with 10 ml of ice-cold PBS. The cells, comprising ~30% macrophages as

Abbreviations used in this paper: BCG, Mycobacterium bovis strain bacillus Calmette-Guérin, substrain M on trehal; C CCP, carbonyl cyanide-m-chlorophenyl hydrazone; LAMP, lysosomal–associated membrane protein; NHS-CF, N -hydroxysuccinimidyl 5-(and 6-)carboxyfluorescein; N ramp, natural resistance–associated macrophage protein; pH e, endosomal pH; pH p, phagosomal pH; SLO, streptolysin O; V-ATPases, vacuolar–type proton ATPases.
wild-type mice were exposed to fluoresceinated bacteria to allow for adherence to occur. After 1 h at 37°C, cells were washed in PBS, then resuspended in Hepes-buffered medium RPMI 1640 containing 10% FCS. Cells (10^9/ml) were then incubated with bacteria as described above. The labeling procedure had no effect on bacterial viability, which was determined by Wright staining, where indicated, or by microscopy. Dead bacteria emit red fluorescence and are detectable under rhodamine optics. Heat-killed bacteria were used for comparison.

Measurement of Phagosomal pH. Measurements of phagosomal pH (pHph) were obtained through the combined application of video microscopy and fluorescence ratio imaging. Suspended peritoneal macrophages from knockout mice were treated with FITC-labeled human holotransferrin (20 μg/ml) for 15 min at 37°C. Cells were then washed in PBS to remove free transferrin and placed in a thermostatted Leiden holder for single-cell imaging as described above. Cells were irradiated at each of the two excitation wavelengths (500BP10 and 440BP10 nm) for 500 ms, and where indicated, were treated with 100 μM concanamycin. Calibration of pHph was achieved using monensin and nigericin as described above.

Immunofluorescence. Immunofluorescence studies were performed on cells from wild-type and knockout mice that had internalized labeled mycobacteria or latex particles. Mycobacteria were labeled with NHS-CF (0.01 mg/ml) as described above. Latex particles were opsonized by incubating in FCS for 1 h at 37°C, washed in PBS to remove unbound opsonin, and added to cells at a density of 10 beads per cell, as described (38). Where indicated, cells were preincubated with FITC-dextran (1 mg/ml) for 15 min at 37°C to label early endosomes. Cells were fixed for 3 h with 4% paraformaldehyde in PBS at room temperature and washed in PBS containing 100 mM glycine for 10 min. The cells were then permeabilized in 0.1% Triton X-100 in PBS for 20 min at 22°C, washed in ice-cold PBS, and blocked with 5% donkey serum in PBS for 1 h at room temperature. For localization of the 39-kD subunit of the V-ATPase, coverslips were then incubated with a 1:100 dilution of the affinity-purified antibody and a 1:600 dilution of Cy3-labeled anti-rat IgG. The specificity of the anti-39-kD subunit antibody was confirmed by preincubating it with a fivefold molar excess of purified fusion protein for 1 h at room temperature, which prevented binding to the cells (not shown). For localization of LAM P-2, coverslips were fixed, permeabilized, and blocked as above then incubated with a 1:4 dilution of anti-LAM P-2 antibody (hybridoma culture supernatant) and a 1:600 dilution of Cy3-labeled anti-rat IgG.

After staining, cells were mounted using Slow Fade (Molecular Probes, Inc.) and sealed with nail polish. Fluorescence was analyzed with a laser confocal microscope (model TCS4D; Leica Instruments GmbH, Heidelberg, Germany) with a ×63 objective. Composite images were assembled and labeled using
Photoshop and Illustrator software (Adobe, Mountain View, CA). All experiments were performed at least four times. Representative confocal images are displayed where appropriate.

Results

Determination of the pH of Phagosomes Containing M. bovis. Single cell spectroscopy was used to measure the pH of phagosomes induced by ingestion of M. bovis in macrophages isolated from Nramp1-expressing (wild-type) and Nramp1−/− mutant mice. Mycobacteria were covalently labeled with fluorescent, pH-sensitive dyes that emitted signals detectable by ratio imaging. Two dyes with different H+ affinities (fluorescein, pKₐ = 6.4, and Oregon green, pKₐ = 4.7) were used in combination, extending the range of reliable pH measurement from ~4.0 to 7.5. The procedure used to label the bacteria had no effect on their viability, as previously determined by Oh et al. (43). Phagocytosis was induced by incubating wild-type or Nramp1−/− macrophages with labeled bacteria for 1 h at 37°C. Fig. 1 illustrates a representative experiment performed in wild-type macrophages. Fig. 1A shows a differential interference contrast (Nomarski) microscopy image of macrophages interacting with live M. bovis. As displayed in the accompanying fluorescence ratio image (Fig. 1B), several of these bacteria (e.g., filled white arrows) are in an acidic environment (note pseudocolor calibration scale on the right), while others (e.g., open arrow) are exposed to near neutral pH. It is likely that the latter are exposed to the extracellular milieu, whereas the acidic bacteria are located within phagosomes. However, Nomarski microscopy could not reliably discriminate between extracellular and intracellular bacteria, nor could all the adherent extracellular bacteria be removed by

Figure 1. Measurement of pHp. Peritoneal macrophages isolated from wild-type Nramp1-expressing mice (strain 129/sv) were allowed to interact with live, fluoresceinated M. bovis, some of which became internalized. The cells were then plated on glass coverslips, mounted in thermoregulated chambers, and visualized using differential interference contrast optics (A), while the fluorescence was measured with alternating excitation at 440 and 490 nm. The fluorescence ratio (B and C) was used for the measurement of the pH in the vicinity of the bacterial surface. After acquiring baseline pH measurements (B), the cells were exposed to 20 mM NH₄Cl and recording was resumed (C). A pseudocolor pH scale is shown to the right. Internalized bacteria, identified by their resting acidic pH and responsiveness to NH₄Cl (filled white arrowheads) and an adherent extracellular bacterium (open arrowhead) are indicated. A typical time course of pH determination in internalized (filled red circles) and adherent bacteria (open circles) are shown in D. The addition of 20 mM NH₄Cl is indicated. Representative of 37 individual experiments.
washing. Therefore, we routinely used three functional criteria to verify that the acidic particles had indeed been internalized. Bacteria within phagosomes were identified not only by their acidic initial pH, but also by the occurrence of a rapid alkalization upon addition of NH$_4$Cl. Preferential permeation of NH$_3$ across the plasma and phagosomal membranes, followed by its protonation in the phagosomal lumen, result in selective alkalinization of intraphagosomal bacteria. Conversely, abrupt changes in the pH of the bathing solution were predicted to affect acutely the pH of extracellular bacteria, but not that of intraphagosomal bacteria. Therefore, we considered the possibility that the intermediate acidification observed was the consequence of a Donnan equilibrium, generated by nondiffusible anionic charges on the bacterial wall. This phenomenon was believed to be responsible for the acidification of normal phagosomes. In susceptible host cells, such ATPases are largely absent from mycobacterial phagosomes, seemingly accounting for their elevated pH. However, although higher than that of other phagosomes, the pH of mycobacterial phagosomes is nevertheless nearly one unit lower than that of other phagosomes, possibly on the bacterial wall. This mechanism of mycobacterial phagosome acidification is believed to be responsible for the differential acidification. V-ATPases are believed to be responsible for the acidification of normal phagosomes. In susceptible host cells, such ATPases are largely absent from mycobacterial phagosomes, seemingly accounting for their elevated pH. However, although higher than that of other phagosomes, the pH of mycobacterial phagosomes is nevertheless nearly one unit lower than that of other phagosomes, possibly on the bacterial wall. This prediction was tested by adding the H$^+$-selective ionophore carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) to macrophages that had internalized live M. bovis. Increasing the passive H$^+$ conductance would be expected to have little effect or even accentuate a pH gradient induced by a Donnan potential. Contrary to this prediction, the...
acidiﬁcation noted in mycobacterial phagosomes from N ramp1−/− macrophages was rapidly dissipated by the protonophore (Fig. 3A), implying that intraphagosomal H+ were not at electrochemical equilibrium. Similarly, CCCP alkalinized the pH of mycobacterial phagosomes from N ramp1-expressing macrophages (Fig. 3A). These ﬁndings indicate that a Donnan equilibrium is not an important contributor to phagosomal acidiﬁcation and that H+ are actively accumulated in the lumen of phagosomes from both normal and N ramp−/− mice, though to different extents.

Fig. 3B shows that, in both types of macrophages, the active accumulation of H+ is sensitive to concanamycin. At the concentration used (100 nM), this inhibitor selectively blocks V-ATPases (45). Therefore, active H+ pumps appear to be functional in the phagosomal membrane of both wild-type and N ramp1−/− mice. This observation agrees with recent observations made in macrophages from C57BL/6J mice (N ramp101293), a strain that is naturally susceptible to mycobacteria (9).

Steady state pHp is dictated by the net result of proton pumping, efflux and metabolic production or consumption, in combination with the endogenous buffering power. Alterations in one or more of these parameters must be responsible for the differences in pHp noted between wild-type and N ramp1−/− macrophages. The contribution of each of these parameters was analyzed.

Phagosomal Buffering Power. In principle, the observed differences in pH between phagosomes from wild-type and N ramp1−/− mice could be attributable to differences in their buffering capacity. To test this possibility, the phagosomal buffering power was measured by pulsing cells that had internalized live M. bovis with small aliquots of NH4Cl (1–2 mM). The change in pHp resulting from entry and protonation of NH4+ was used to compute the buffering power. Because buffering capacity can vary with pH, care was taken to make the comparisons in the same range of pHp. This is particularly important because the initial pHp of the two types of macrophages is different. As shown in Table 1, the buffering capacity of mycobacterial phagosomes in the 6.5 to 6.8 range was similar in both wild-type and N ramp1−/− macrophages. Therefore, other parameters must be responsible for their differential steady state pHp.

Effects of the Electrogenic Na+ Pump on the Proton motive Force. Because the process of H+ pumping by the V-ATPase is rheogenic, it is affected by changes in the transmembrane potential. In some systems, the rate or extent of acidiﬁcation of endosomes has been shown to be modulated by the parallel activity of the Na+ pump, an electrogenic ATPase (46, 47). Therefore, we considered the possibility that enhanced Na+ pump activity contributed to the reduced acidiﬁcation of N ramp1−/− phagosomes. To test this hypothesis, ouabain was added to the medium before phagocytosis and was present throughout. High (millimolar) concentrations of the drug were used to ensure inhibition of the pump, which in rodents has low afﬁnity for the glycoside. Ouabain had no signiﬁcant effect on pHp (not described for Fig. 1 (see Materials and Methods) in peritoneal macrophages obtained from wild-type mice (open symbols) and N ramp1−/− mice (ﬁlled symbols). Phagosome formation was induced using live, ﬂuoresceinated M. bovis. After acquiring baseline measurements, 10 μM CCCP (A) or 100 nM concanamycin (B, CCM) was added to both samples. Data are representative of ﬁve determinations.

Table 1: Physiological Characteristics of Phagosomes from N ramp1 Wild-type (N ramp1+/+ ) and N ramp1−/− Mice

| Buffering power* (mmol/pH/liter) | 18 ± 2 | 16 ± 4 |
|---------------------------------|--------|--------|
| H+ consumption by RO O1 (ΔpHp) | 0.2 ± 0.05 | 0.3 ± 0.1 |
| Net H+ efflux (pH/min)          | 0.2 ± 0.03 | 0.2 ± 0.02 |
| Counterion conductance (ΔpHp)   | 0.3 ± 0.03 | 0.2 ± 0.04 |

* M acrophages from wild-type and N ramp1−/− mice that had internalized live M. bovis were pulsed with small aliquots of NH4Cl (1–2 mM). The change in pHp resulting from entry and protonation of NH4+ was used to compute the buffering power, which was compared in the 6.5–6.8 range in both cell types.

† M acrophages from both strains that had internalized live M. bovis were treated with the ﬂavoprotein inhibitor diphenylene iodonium (3 μM), and steady state pHp was reached. Results are expressed as pHp after addition minus pHp before addition.

‡ Net phagosomal H+ efflux was induced in macrophages from wildtype and N ramp1−/− mice that had internalized live M. bovis by addition of concanamycin (100 nM). Because the buffering power of both types of phagosomes is similar, the ﬂuxes are expressed as the rates of change of pH per unit time, and were made at comparable pHp.

§ Cells from both strains were allowed to internalize live M. bovis in a K+–rich medium. After steady state pHp was reached, cells were treated with valinomycin (10 μM), a conductive K+ ionophore. Results are expressed as pHp after addition minus pHp before addition.
shown). Therefore, it is unlikely that differential electrogenic effects of the Na⁺/K⁺-ATPase contribute to the difference in pHₚ noted between wild-type and Nramp⁻/⁻ macrophages.

H⁺ consumption by Superoxide Dismutation. It has been proposed that superoxide radicals, formed within the phagosome by the NADPH oxidase, consume H⁺ during the dismutation reaction, forming hydrogen peroxide and water (48). Therefore, we considered the possibility that Nramp affected pHₚ indirectly by altering the rate of superoxide production or dismutation. Macrophages from wild-type and Nramp⁻/⁻ mice that had internalized live M. bovis were treated with the flavoprotein inhibitor diphenylene iodonium, at concentrations known to produce complete inhibition of the NADPH oxidase. As shown in Table 1, the inhibitor had no effect on steady state pHₚ in macrophages from either strain. This finding suggests that because macrophages generate superoxide at relatively low rates unlike neutrophils (49), the dismutation reaction does not contribute significantly to net H⁺ consumption in the phagosome. Hence, superoxide dismutation cannot account for the differential behavior of the two types of macrophages.

Phagosomal H⁺ (Equivalent) "Leak". Steady state pHₚ is established by the differential between the rates of H⁺ influx (pumping) and efflux (leakage of H⁺ and H⁺-equivalent species). Because both wild-type and Nramp⁻/⁻ macrophages seem to share the same pumping mechanism, we considered the possibility that leaks of different magnitude might account for their different pHₚ. Net H⁺ efflux was induced by inhibiting the V-ATPases with concanamycin, as in Fig. 3. Because the buffering power of both types of phagosomes is similar, the fluxes are expressed as the rates of change of pH per unit of time. The measurements were made at comparable pHₚ to ensure that any differences in the fluxes reflected the permeability to H⁺ and not its driving force. As shown in Table 1, proton leakage rates were found to be similar in both wild-type and Nramp⁻/⁻ mice. Therefore, the difference in the steady state pHₚ between the two types of mice is probably due to differential influx (pumping).

ATP-dependent H⁺ Pumping. At steady state, H⁺ pumping is obscured by H⁺ efflux (leakage) of equal magnitude and opposite direction. The leak is highest at acidic pHₚ, when the force driving efflux is greater. Conversely, the activity of the pump diminishes as the lumen becomes acidic. Therefore, to assess the ability of phagosomes to pump H⁺ encumbered minimally by leakage, we measured the rate of initial acidification, near neutral pHₚ. For this purpose, we first dissipated the H⁺ gradient by depletion of cytosolic ATP, using SLO to permeabilize the plasma membrane. This antibiotic forms large pores in the plasma membrane, without permeabilizing internal organelles including the phagosome (50, 51). As shown in Fig. 4 A, upon addition of SLO to cells suspended in medium devoid of ATP and Mg²⁺, the phagosomal acidification dissipates within minutes. Importantly, this pH change was not observed when the medium contained ATP and Mg²⁺ (data not shown), indicating that it is not a consequence of direct

![Figure 4](image-url)
phagosomal permeabilization by SLO. Instead, neutralization of pH<sub>p</sub> appears to be due largely to washout of ATP and Mg<sup>2+</sup>, the substrates required by the V-ATPase, since acidification was restored by their readdition. Accordingly, the reacidification was virtually eliminated by simultaneous addition of concanamycin (Fig. 4A).

As shown in Fig. 4B, the initial rate and extent of acidification recorded upon reintroduction of ATP and Mg<sup>2+</sup> were significantly greater in M. bovis phagosomes of wild-type mice than in their N<sup>ramp1</sup>−/− counterparts. The results of five similar experiments are summarized in Fig. 4C. From these observations, we conclude that elimination of N<sup>ramp1</sup> results in a decreased ability to pump H<sup>+</sup> into mycobacterial phagosomes. This is most simply explained by a reduction in the number of V-ATPases, but other possible explanations exist (see below).

Counterion Permeability. To maintain electroneutrality in the course of net translocation of H<sup>+</sup> by the V-ATPase, stoichiometric movement of counterions is required. It has been postulated that the rate of acidification can be limited not by the ability of the ATPases to pump H<sup>+</sup>, but by the passive permeability of the membrane to counterions (52). Accordingly, the difference in the pumping rates noted between wild-type and N<sup>ramp1</sup>−/− mice could be attributed to reduced counterion permeability in the case of the latter. Two lines of evidence argue against this possibility. First, addition of CCCP to the N<sup>ramp1</sup>−/− macrophages rapidly increased pH<sub>p</sub> (Fig. 3). Because in the steady state H<sup>+</sup> pumping and leakage are identical, dissipation of the acidification by CCCP indicates that the rate of leakage greatly exceeded pumping. The excess leakage of H<sup>+</sup> induced by the protonophore must have been supported by a flux of counterions that was greater than that needed to operate the pump in the steady state, implying that the resting conductance is greater than necessary to support the basal rate of pumping, i.e., that counterion permeability is not rate limiting.

One might argue that the conductive pathway may display rectification, i.e., that the counterion permeability is greater in one direction than in the other. A second type of experiment was devised to consider this possibility. Cells from both strains were allowed to internalize live M. bovis in a K<sup>+</sup>-rich medium. After steady state pH<sub>p</sub> was reached, cells were treated with valinomycin, a conductive K<sup>+</sup> ionophore. Under these conditions, cations are readily able to exit the phagosomal lumen, providing an adequate counterion for H<sup>+</sup> pumping. As shown in Table 1, addition of valinomycin had minimal effects on pH<sub>p</sub> in both strains of cells. These findings confirm that counterion conductance is not limiting to phagosomal acidification and therefore cannot account for the differential acidification seen in wild-type and N<sup>ramp1</sup>−/− macrophages.

Phago-lysosomal Fusion in Wild-type and N<sup>ramp1</sup>−/− M. bovis macrophages. In general, phagosomes are believed to acquire H<sup>+</sup> pumps after fusion with late endosomes/lysosomes, which are a rich source of V-ATPases (9, 10, 32, 53). In permissive cells live mycobacteria are known to impair phagosome fusion to V-ATPase-containing vesicles (11), likely accounting for the inability of the phagosomes to acidify normally. Therefore, it is conceivable that expression of N<sup>ramp1</sup> restores the ability of the phagosomes to interact with late endosomes and lysosomes. This possibility was assessed by quantifying by immunofluorescence the insertion of LAM-P-2, a marker of the membranes of late endosomes and lysosomes, into the phagosomal membrane of wild-type and N<sup>ramp1</sup>−/− macrophages. Phagosomes induced by live M. bovis or latex particles were compared in both types of cells. As illustrated in Fig. 5, C, F, and H, both types of macrophages accumulated LAM-P-2 on the membrane of phagosomes formed by internalization of latex beads. In contrast, recruitment of LAM-P-2 to the membrane of M. bovis phagosomes was significantly impaired in N<sup>ramp1</sup>−/− macrophages (Fig. 5, D, E, and G; overall 7% positive) when compared with wild-type cells analyzed under similar conditions (Fig. 5, A, B, and G; overall 26% positive). Therefore, increased fusion of mycobacterial phagosomes with late endosomes/lysosomes in wild-type macrophages may explain their increased V-ATPase activity and greater acidification.

Delivery of V-ATPases to Mycobacterial Phagosomes from N<sup>ramp1</sup>−/− M. bovis. Although increased fusion to late endosomal/lysosomal compartments provides an explanation for the increased V-ATPase activity in wild-type compared with N<sup>ramp1</sup>−/− cells, it fails to account for the presence of V-ATPases and partial acidification seen in N<sup>ramp1</sup>-negative phagosomes containing live mycobacteria. It has previously been demonstrated that, although mycobacterial phagosomes of permissive cells do not fuse with late endosomes and lysosomes (11, 12, 54), they retain the capacity to interact with early and recycling endosomes (9, 13, 55). Importantly, in other systems these compartments can accumulate H<sup>+</sup> in a concanamycin-sensitive manner (9, 53, 56), and may thus represent a source of V-ATPases for mycobacterial phagosomes in N<sup>ramp1</sup>−/− mice. Therefore, we investigated whether fusion with early endosomes was responsible for delivery of V-ATPases to mycobacterial phagosomes in N<sup>ramp1</sup>−/− mice. The results of these studies are summarized in Fig. 6. M acrophages from N<sup>ramp1</sup>−/− mice were allowed to internalize fixable Texas red-dextran (a fluid phase marker) for 15 min, a period sufficient to direct this dye to the early endosomal compartment (57; Fig. 6A). These macrophages were then fixed, and the subcellular distribution of the V-ATPase was detected with an antibody raised to its 39-kD subunit. The V-ATPases demonstrated a punctate, vesicular pattern suggestive of endomembrane localization (Fig. 6B). As shown in Fig. 6C (superimposed images from A and B), there was significant, yet incomplete, colocalization of the 39-kD subunit with the fluid phase marker, suggesting the presence of V-ATPases in the early endosomal compartment. To assess the propensity of M. bovis-containing phagosomes to fuse with early endosomes, macrophages that had internalized mycobacteria were treated with Texas red-dextran as above (Fig. 6D and E). In 48% of instances, the mycobacterial phagosome was found to be outlined by the endosomal marker,
confirming that phagosome-endosome fusion had occurred in these cells (Fig. 6F).

Even though most of the material internalized by fluid phase endocytosis recycles back to the surface (58), and despite the use of comparatively short pulses, a small fraction of the fluorescence may have reached late endosomes. Therefore, it was essential to confirm that early/recycling endosomes of Nramp1−/− mutant mice expressed functional V-ATPases. Therefore, macrophages from these mice were treated with FITC-labeled transferrin, which enters the cells via clathrin-dependent endocytosis and accumulates in the early/recycling endosomal compartment without ever reaching late endosomes (59, 60). The pH of this compartment was determined to be more acidic than the cytoplasm (6.6 ± 0.02, Fig. 6G), in agreement with values determined in other cell types (60, 61). Upon addition of concanamycin, the acidification was rapidly dissipated (Fig. 6G), confirming that the accumulation of H+ within early/recycling endosomes is mediated by V-ATPases. These data suggest that mycobacterial phagosomes from Nramp1−/− mice can acquire V-ATPases as a result of fusion with early endosomes.

Discussion

This study determined that phagosomes containing mycobacteria (M. bovis) in wild-type mice are significantly more acidic than those formed in macrophages from Nramp1−/− mutant mice (Fig. 2). This difference in pH could conceivably account for host resistance to mycobacterial infection by several mechanisms. First, the enhanced acidification is expected to have a direct bacteriostatic effect, since mycobacteria grow optimally in the pH range 6.5–7.9 (43). In addition, the acidic milieu can activate microbiocidal enzymes delivered to the phagosome during fusion with lysosomes (31). Also, the low pH favors the protonation of nitrite to nitrous acid. Dismutation of the latter promotes the formation of reactive nitrogen species that are effective bactericidal agents (62). The latter can promote the formation of reactive species and is therefore a more effective bactericidal agent. Finally, the increased acidification would promote the dismutation of superoxide to hydrogen peroxide, which in turn functions as the substrate for myeloperoxidase delivered by phago-lysosomal fusion. Of note, the defect in phagosomal acidification noted in Nramp1−/− mice was also detected in macrophages from C57BL/6 mice, a strain susceptible to M. bovis infection and that bears a naturally occurring mutant G169D allele at the Nramp1 locus that prevents expression of the protein (27, 28). Together, these data suggest that the role of Nramp1 in restricting the replication of intracellular pathogens such as mycobacteria is at least in part by alteration of pHp.

Several possible mechanisms could account for the differences in pHp between normal and Nramp1−/− macrophages, and these were each investigated in turn. It has previously
been speculated that the generation of intraphagosomal superoxide could consume a significant amount of H+ through dismutation during the course of hydrogen peroxide production. However, in macrophages this reaction is unlikely to contribute significantly to the higher pHp of N ramp1<sup>−/−</sup> phagosomes, as inhibition of the NADPH oxidase had no discernible effect on pHp (Table 1). Likewise, phagosomal buffering power, counterion conductance and the rate of H+ (equivalent) leak were found to be comparable between the two types of cells, and are hence unlikely to explain the observed difference in the extent of acidification (Table 1). Rather, the lower pHp of wild-type macrophages was most likely due to increased rates of active proton accumulation in mycobacterial phagosomes. Support for this notion was obtained using cells treated with SLO (Fig. 4): selective permeabilization of the plasma membrane by this toxin resulted in reversible inhibition of V-ATPase activity, caused by depletion of cytosolic ATP/Mg. Accordingly, readdition of ATP/Mg<sup>2+</sup> induced rapid phagosomal acidification at a rate significantly greater in wild-type mice than in the N ramp1<sup>−/−</sup> counterparts. These data strongly support the conclusion that mycobacterial phagosomes in N ramp1-expressing macrophages are more acidic due to increased activity of V-ATPases.

The results of several laboratories, including our own (9, 11, 12), demonstrate a paucity of ATPases in mycobacterial phagosomes, as evaluated by immunological means. On the other hand, the functional determinations presented here indicate that concanamycin-sensitive proton pumping is in fact detectable in such phagosomes. To reconcile these observations, we considered a model whereby V-ATPases could induce acidification of the phagosomal lumen while being physically absent from the phagosomal membrane (9). Proton equivalents could be delivered to the phagosomal lumen by acid carrier vesicles that are derived from endosomes (or other acidic organelles), yet are themselves devoid of V-ATPases. Though unable to interact with lysosomes, the mycobacterial phagosome could nevertheless fuse with such vesicles and become partially acidic. Continued vesicular traffic to the phagosome would explain the sustained, concanamycin-sensitive acidification in a compartment that lacks ATPases. Although attractive, this model appears untenable, in view of the data obtained using SLO-permeabilized cells. Fusion of vesicular compartments within cells is

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**Figure 6.** Localization of V-ATPases in early endosomes and in phagosomes. (A–C) Macrophages obtained from N ramp1<sup>−/−</sup> mice were allowed to internalize Texas red-labeled fixable (1 mg/ml) dextran for 15 min at 37°C. The cells were then fixed, permeabilized, and incubated with an affinity-purified polyclonal antibody against the 39-kD subunit of the V-ATPase, followed by an FITC-labeled secondary antibody. (A) Localization of Texas red-labeled dextran; (B) localization of the V-ATPase subunit; (C) areas of overlap between the dextran and the ATPase. (D–F) Macrophages obtained from N ramp1<sup>−/−</sup> mice were allowed to internalize live, fluoresceinated M. bovis and were subsequently incubated with Texas red-labeled dextran for 15 min at 37°C. The cells were then fixed and visualized by Nomarski (D) and confocal immunofluorescence microscopy. (E) Localization of fluoresceinated bacteria; (F) distribution of Texas red-labeled dextran. Images are representative of at least five experiments of each kind. (G) Macrophages obtained from N ramp1<sup>−/−</sup> mice were allowed to internalize FITC-labeled human holotransferrin (20 μg/ml), and pH was measured using single-cell imaging. Where indicated, concanamycin (100 nM) was added. Data represent means ± SEM of three separate experiments.
known to require various cytosolic cofactors, including GTP, NSF, and SNAPs, which are expected to wash out of cells perforated by SLO. Nevertheless, in such cells phagosomal acidification was effectively reactivated by the mere readdition of Mg\(^{2+}\) and ATP, implying that vesicular fusion was not required for this process.

A more likely explanation for the observed acidification is therefore that V-ATPases are in fact present and functional on the phagosomal membrane itself, though at a comparatively low density that is difficult to detect by chemical or immunological means. What then is the likely source of such phagosomal ATPases? Recent reports by the laboratories of Russell (13) and Horowitz (55) demonstrated that mycobacterial phagosomes can fuse and remain in dynamic equilibrium with (components of) the early and recycling endosomal compartments of susceptible macrophages. Such compartments could in principle deliver the proton pumps to the phagosome, as suggested by our functional and immunofluorescence data. As shown in Fig. 6, early/recycling endosomes of N ramp\(^{-/-}\) macrophages acidify in a concanamycin-sensitive manner, confirming that they possess V-ATPases. Partial colocalization with the 39-kD subunit of the pump supports this notion. Moreover, in confirmation of the earlier results (13, 16), early endosomes were found to be capable of fusing with phagosomes containing live mycobacteria (Fig. 6).

The enhanced V-ATPase activity and lower pH, observed in cells from mice expressing N ramp likely reflects the additional ability of these phagosomes to fuse readily with late endosomes and/or lysosomes (63; Fig. 5 G), a process that is seemingly impaired by the bacteria in the N ramp\(^{-/-}\) mice. The observations can be most simply explained by postulating that the arrest in phagosomal maturation induced by internalized live mycobacteria in susceptible mice is circumvented by N ramp1 (9, 11, 54, 55). What is the mechanism whereby N ramp1 facilitates phagolysosomal interaction? N ramp1, which resides in the membrane of lysosomes in quiescent cells, may itself act as a fusogen, in a manner analogous to SNARE or rab proteins, thus promoting interaction with the maturing phagosome (38, 64–66). No direct evidence in support of this mechanism has been provided and there is no homology between the structure of N ramp1 and those of proteins involved in vesicular docking and fusion. Rather, recent findings indicate that members of the N ramp family function as transporters for divalent cations (67). The N ramp2 protein is closely related to N ramp1, sharing a high degree of primary sequence homology (77% similarity) and very similar structural features (68). Recent electrophysiological studies in Xenopus oocytes demonstrated that N ramp2 functions as a divalent cation transporter, translocating Fe\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Cd\(^{2+}\), and several other cations (69). N ramp2-mediated transport is pH dependent, and the divalent cations seem to be cotransported with H\(^{+}\) (69). In parallel, it was reported that mice bearing a mutation at the N ramp2 locus show severe microcytic anemia, leading to the conclusion that N ramp2 is the major transferrin-independent Fe\(^{2+}\) uptake system of the intestine (70). In addition, the yeast Saccharomyces cerevisiae has three N ramp proteins (Smf1, Smf2, Smf3) that share strong homology to the mammalian family members (47% similarity), one of which (Smf1) has been demonstrated to be a Mn\(^{2+}\) transporter (67). We have recently shown that, despite their large evolutionary distance, N ramp2 can complement a double smf1/2 yeast mutant and restore growth on EGTA and at alkaline pH (71). This conservation of function among distant N ramp family members strongly suggests that N ramp1 may also be a divalent cation transporter, acting at the lysosomal and/or phagosomal membrane. In this event, the transported substrate could conceivably affect phago-lysosome fusion. Interestingly, in vitro studies have recently shown that endosome-lysosome fusion is dependent upon the availability of Zn\(^{2+}\) (72). Moreover, calcium is known to be essential for phago-lysosomal fusion in neutrophils (73), though not in macrophages (74). Inhibition of mycobacterial replication may also result from the N ramp1-mediated elimination of metals that are essential for their growth or for protection against cellular microbicidal agents. Regardless of the mechanism, it is important to point out that the observed effects of N ramp1 on phagosomal maturation are limited to phagosomes containing live mycobacteria, since the pH and fusogenic activity of phagosomes containing dead bacteria or latex beads appear to be similar in wild-type and N ramp1\(^{-/-}\) macrophages (Fig. 5). Therefore, it is reasonable to propose that N ramp1 antagonizes the effect of a factor produced by live mycobacteria, which interferes with phagosomal maturation.

In summary, this study shows that the unrestricted replication of intracellular pathogens in vivo caused by elimination of N ramp1 function in a N ramp1\(^{-/-}\) host is concomitant to impaired acidification of phagosomes containing live mycobacteria. The presence of the N ramp1 protein appears to bypass the arrest in phagosomal maturation typically observed in susceptible strains by enhancing the capacity of the mycobacterial phagosome to fuse to late endosomes and/or lysosomes, leading to increased V-ATPase activity at the phagosomal membrane, and consequently enhanced phagosomal acidification. Studies are now ongoing to identify the putative substrates translocated by N ramp1 that may modulate phagosomal maturation.

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References

1. Young, P. 1996. White House to expand response to infectious diseases. Am. Soc. Microbiol. News 62:450–451.

2. Berkelman, R.L., R.T. Bryan, M.T. Osterholm, J.W. Leduc, and J.M. Hughes. 1996. Infectious disease surveillance: a crumbling foundation. Science 264:368–370.

3. Bloom, B.R. 1992. Tuberculosis back to a frightening future. Nature 358:338–340.

4. Kaye, K., and T.R. Frieden. 1996. Tuberculosis control: the relevance of classical principles in an era of acquired immunodeficiency syndrome and multidrug resistance. Epidemiologic Rev. 18:52–63.

5. Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science 264:375–382.

6. Neu, H.C. 1992. The crisis in antibiotic resistance. Science 257:1064–1072.

7. Heubner, R.E., and K.G. Castro. 1995. The changing face of Mycobacterium leprae. Trends Genet. 11:539–552.

8. Young, D.B., and K. Duncan. 1997. Prospects for new interventions in the treatment and prevention of Mycobacterial disease. Annu. Rev. Microbiol. 51:467–574.

9. Shackelford, R.W., D. Maleo, K. Vogan, E. Skamene, and P. Gros. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. Curr. Opin. Immunol. 5:378–388.

10. Schurr, E., K. Vogan, S. Vidal, J. Hu, M. Cellier, E. Schurr, A. Fuks, N. Burnstead, K. Morgan, and P. Gros. 1994. haplotype mapping and sequence analysis of the mouse N r m gene predict susceptibility to infection with intracellular parasites. Genomics. 23:51–61.

11. Skamene, E., S. Vidal, and P. Gros. 1998. Infection genomics: N r m as a major determinant of natural resistance to intracellular infections. Annu. Rev. Med. 49:275–287.

12. Goto, Y., E. Buschman, and E. Skamene. 1989. Regulation of host resistance to Mycobacterium tuberculosis by the Bcg gene. Immunogenetics 30:218–221.

13. Stach, J.L., P. Gros, A. Forget, and E. Skamene. 1984. Phenotypic expression of genetically-controlled natural resistance to Mycobacterium bovis (BCG). J. Immunol. 130:218–221.

14. Denis, M., A. Forget, M. Pelletier, F. Gervais, and E. Skamene. 1990. Killing of Mycobacterium smegmatis by macrophages from genetically susceptible and resistant mice. J. Leukocyte Biol. 47:25–30.

15. Vidal, S.M., E. Pinner, P. Lepage, S. Gauthier, and P. Gros. 1996. Natural resistance to intracellular infections. N r m encodes a membrane phosphoglyceroprotein absent in macrophages from susceptible (N r m 169) mouse strains. J. Immunol. 157:3559–3568.

16. Vidal, S., M.L. Tremblay, G. Govoni, G. Gauthier, G. Sebastiani, D. Maleo, E. Skamene, M. Oulivier, S. Jotthy, and P. Gros. 1995. The Its/Ityb locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the N r m gene. J. Exp. Med. 182:655–666.

17. Govoni, G., S. Vidal, G. Gauthier, E. Skamene, D. Maleo, and P. Gros. 1996. The Bcg Its/Ityb locus: genetic transfer of resistance to infections in C57BL/6j mice transgenic for the N r m mouse strain. J. Immunol. 156:2846–2853.

18. Greenberg, S., S.C. Silverstein. 1993. Phagocytosis In Fundamental Immunology. W.E. Paul, editor. Raven Press, New York. 941–964.

19. Lukacs, G.L., O.D. Roststein, and S. Grinstein. 1990. Phagocytosis is mediated by a vacuolar-type H+-ATPase in murine macrophages. J. Biol. Chem. 265:21099–21107.

20. Verkruyse, L.A., and S.L. Horn. 1996. Lysosomal targeting of palmitoyl-protein thioesterase. J. Biol. Chem. 271:362.

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35. Clemens, D.L., and M.A. Horwitz. 1995. Characterization of the M yobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. J. Exp. Med. 181:257–270.

36. Colletie, J., D. M cGreer, R. Crawford, F. Chubb, and R.D. Sandin. 1956. Synthesis of some cyclic iodonium salts. J. Am. Chem. Soc. 78:3819–3820.

37. Chen, J.W., T.L. Murphy, M.C. Willingham, I. Pastan, and J.T. August. 1985. Identification of two lysosomal membrane glycoproteins. J. Cell Biol. 101:85–95.

38. H ackam, D.J., O.D. R otstein, M.K. Bennett, A. K lip, S. G rinstein, and M.F. M aionio. 1996. Characterization and subcellular localization of target membrane soluble NSF attachment protein receptors (t-SNAREs) in macropahges. Syntaksins 2, 3 and 4 are present on phagosomal membranes. J. Immunol. 156:4377–4383.

39. F orget, A., E. Skamene, P. Gros, A.C. M ialhe, and R. T urcotte. 1981. Differences in response among inbred mouse strains to infection with small doses of M yobacterium bovis BCG. Infect. Immun. 32:42–47.

40. H ackam, D.J., O.D. R otstein, W.J. Zhang, A.D. Schreiber, and S. G rinstein. 1997. Rho is required for the initiation of calcium transients and phagocytosis by Fc receptors in macrophages. J. Exp. Med. 186:955–966.

41. G rinstein, S., and W. F uruya. 1986. Characterization of the amiloride sensitive Na+/H+ antiport of human neutrophils. A.m.J. Physiol. 250:283–291.

42. R oos, A., and W.F. B oron. 1981. Intracellular pH. Physiol. Rev. 61:296–434.

43. O h, Y.K., and R.M. S traubinger. 1996. Intracellular fate of M yobacterium avium: use of dual label spectrofluorimetry to investigate the influence of bacterial viability and osmoporation on phagosomal pH and phagosome-lysosome interaction. Infect. Immun. 64:319–325.

44. T exeira, H.C., M.E. M unk, and S.H. K auffman. 1995. Frequencies of IFN gamma and IL-4 producing cells during infection of M yobacterium avium BCG in two genetically susceptible mouse strains: role of alpha/beta T cells and NK1.1 cells. Immunol. Lett. 55:15831–15836.

45. V ia, L.E., D. D erstic, R.J. U lmer, N.S. H iber, L.A. Huber, and V. D eretic. 1997. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J. Biol. Chem. 272:13326–13331.

46. C lemens, D.L., and M.A. H orwitz. 1996. The M yobacterium tuberculosis phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. J. Exp. Med. 184:1349–1355.

47. Gluck, S.L. 1993. The vacuolar H+ -ATPases: versatile proton pumps participating in constitutive and specialized functions of eukaryotic cells. Rev. Cytol. 137:105–137.

48. M ellman, I. 1996. Membranes and sorting. Curr. Opin. Cell Biol. 8:497–498.

49. M ellman, I. 1996. Endocytosis and molecular sorting. Annu. Rev. Cell Biol. 12:575–625.

50. D autry-Varsat, A., A.A. C iechanover, and H.F. Lodish. 1983. pH and the recycling of transferrin during receptor mediated endocytosis. Proc. Natl. Acad. Sd. USA. 80:2258–2262.

51. Y amahiro, D.J., B. T ycko, S.R. F luss, and F.R. M axfield. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. Cell. 37:789–800.

52. M ukherjee, S., R.N. G hosh, and F.R. M axfield. 1997. Endocytosis. Physiol. Rev. 77:759–804.

53. S tiehr, D.J., and C.F. N athan. 1989. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. 169:1543–1555.

54. de C hastellier, C., C. F rehel, C. O ffredo, and E. Skamene. 1993. Implication of phagosome-lysosome fusion in restriction of M yobacterium avium growth in bone marrow macrophages from genetically resistant mice. Infect. Immun. 61:3775–3784.

55. S udhof, T.C., P. D e C amilli, H. N iemann, and R. Jahn. 1993. Membrane fusion machinery: insights from synaptic proteins. C.ell. 75:1–4.

56. R othman, J.E. 1994. Mechanisms of intracellular protein transport. Nature. 372:55–63.

57. N ovick, P., and B. B rennwald. 1993. Friends and family: the role of the R ab GT Pases in vesicular traffic. C.ell. 75:597–601.

58. S upek, F., L. S upekova, H. Nelson, and N. N elson. 1996. A yeast manganese transporter related to the macrophage proton pump involved in conferring resistance to mycobacteria. Proc. Natl. Acad. Sd. USA. 93:5105–5110.

59. G ruenheid, S., M. C ellier, S. Vidal, and P. G ros. 1995. Identification and characterization of a second mouse N ramp gene. G enomics. 25:514–525.

60. G unshin, H., B. M ackenzie, U.V. B erry, Y. G unshin, M.F. R omero, W.F. B oron, S. N usberger, J.L. G ollan, and M.A. H ediger. 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature. 388:482–488.

61. F leming, M.D., C.C. T renor, M.A. S u, D. F oernzler, D.R. B eier, W.F. D etrich, and N.C. A ndrews. 1997. Microcytic
anemia mice have a mutation in Nramp2, a candidate iron transporter. Nat. Genet. 16:383-386.
71. Pinner, E., S. Gruenheid, M. Raymond, and P. Gros. 1997. Functional complementation of the yeast divalent cation transporter family SMF by NRAMP2, a member of the mammalian natural resistance associated macrophage protein family. J. Biol. Chem. 272:28933-28938.
72. Aballay, A., M.N. Sarrouf, M.I. Colombo, P.D. Stahl, and L.S. Mayorga. 1995. Zn\(^{2+}\) depletion blocks endosome fusion. Biochem. J. 312:919-923.
73. Stendahl, O., K.-H. Krause, J. Krischer, P. Jerstrom, J.-M. Theler, R.A. Clark, J.-L. Carpentier, and D.P. Lew. 1994. Redistribution of intracellular Ca\(^{2+}\) stores during phagocytosis in human neutrophils. Science. 265:1439-1441.
74. Zimmerli, S., M. Majeed, M. Gustavsson, O. Stendahl, D.A. Sanan, and J.D. Ernst. 1996. Phagosome-lysosome fusion is a calcium-independent event in macrophages. J. Cell Biol. 132:49-61.