Cellular Microbiology (2001) **3**(12), 773–784

Microreview

SLC11A1 (formerly NRAMP1) and disease resistance

Jenefer M. Blackwell,* Tapasree Goswami, Carlton A. W. Evans, Dean Sibthorpe, Natalie Papo, Jacqueline K. White, Susan Searle, E. Nancy Miller, Christopher S. Peacock, Hiba Mohammed and Muntaser Ibrahim
Cambridge Institute for Medical Research, University of Cambridge School of Clinical Medicine, Wellcome Trust/ MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, UK.

Introduction

Slc11a1 (formerly Nramp1) has many pleiotropic effects on macrophage (mϕ) activation, including regulation of the CXC chemokine KC, interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), major histocompatibility complex (MHC) class II molecules, tumour necrosis factor α (TNFα), nitric oxide (NO) release, L-arginine flux, oxidative burst and tumoricidal as well as antimicrobial activity (reviewed by Blackwell and Searle, 1999; Blackwell *et al*., 2000). A naturally occurring Gly→Asp mutation at amino acid 169 of Slc11a1 makes mice as susceptible to *Leishmania donovani*, *Salmonella typhimurium* and *Mycobacterium bovis* as gene-disrupted mice (Vidal *et al*., 1995). Hence, the mutation is a functional null. This mutation also confers susceptibility to a range of other pathogens in mice, including *Mycobacterium lepraemurium* (Brown *et al*., 1982; Skamene *et al*., 1984), *Mycobacterium intracellulare* (Goto *et al*., 1989), *Toxoplasma gondii* (Blackwell *et al*., 1994), *Candida albicans* (Puliti *et al*., 1995) and *Leishmania infantum* (Leclercq *et al*., 1996). In man, *SLC11A1* is linked or associated with multiple infectious (Shaw *et al*., 1997; Abel *et al*., 1998; Bellamy *et al*., 1998; Marquet *et al*., 1999; Cervino *et al*., 2000; Gao *et al*., 2000; Greenwood *et al*., 2000; Ryu *et al*., 2000; Mohamed *et al*., 2001) and autoimmune (Shaw *et al*., 1996; Hofmeister *et al*., 1997; Esposito *et al*., 1998; Maliarik *et al*., 2000; Sanjeevi *et al*., 2000; Singal *et al*., 2000; Yang *et al*., 2000) diseases. The infectious diseases include viral (HIV), bacterial (tuberculosis, leprosy, meningococcal meningitis) and protozoan (visceral leishmaniasis) pathogens. The autoimmune diseases include rheumatoid arthritis, juvenile rheumatoid arthritis, diabetes, sarcoidosis and Crohn's disease. Mutation in the closely related *Slc11a2* (*Nramp2*) gene causes microcytic anaemia in mice (Fleming *et al*., 1997), but disease association in man has not been reported. Slc11a1 and Slc11a2 are polytopic integral membrane proteins with 10–12 putative membrane-spanning domains (Vidal *et al*., 1993; Gunshin *et al*., 1997). In both, the natural functional null mutation occurs in transmembrane domain 4 (Vidal *et al*., 1993; Fleming *et al*., 1997). Both Slc11a1 and Slc11a2 have protein kinase C (PKC) binding sites (Vidal *et al*., 1993; Barton *et al*., 1994; Gruenheid *et al*., 1995), but only Slc11a1 has a Pro–Ser-rich N-terminus (Barton *et al*., 1994). Here, we review current knowledge on the evolution, function and roles of Slc11a1/SLC11A1 in disease.

Received 28 May, 2001; revised 20 July, 2001; accepted 25 July, 2001. *For correspondence. E-mail jmb37@cus.cam.ac.uk; Tel. (+44) 122 333 6947; Fax (+44) 122 333 1206.

Localization and tissue distribution

Slc11a1 localizes to membranes of late endosomes and lysosomes in mϕ (Gruenheid *et al*., 1997; Searle *et al*., 1998), but not to early endosomes (Gruenheid *et al*., 1997). This is consistent with the presence of endocytic targeting signals in the 5′ and 3′ ends of Slc11a1 (Atkinson *et al*., 1997) and suggests that it targets directly from the trans-Golgi network (TGN) to the endosomal compartment rather than becoming incorporated into the phagosome membrane as part of the phagocytic process. Mutant mϕ express a protein recognized by both anti-N-terminal and anti-C-terminal anti-Slc11a1 antibodies, but the protein is expressed at lower levels in mature bone marrow-derived mϕ from mutant C57BL/10ScSn mice compared with their wild-type congenic B10.L-Lsh^r counterparts (Searle *et al*., 1998). Expression at the mRNA and protein (Searle *et al*., 1998) levels is upregulated to an equivalent degree in mutant and wild-type mϕ after treatment with lipopolysaccharide and interferon (IFN)-γ. This is accompanied by an increased proportion of Slc11a1 gold labelling in electron-dense lysosomes compared with the electron-lucent late endosomal compartment (Searle *et al*., 1998), but the functional significance of this redistribution of the protein has not been addressed. Slc11a1 targets to *Leishmania* or *Mycobacterium avium* phagosomes (Searle *et al*., 1998). Vesicles recognized by anti-Slc11a1 antibodies rapidly migrate to fuse with *M. avium*-containing phagosomes in resting wild-type but not mutant bone marrow-derived mϕ (Fig. 1; Searle *et al*., 1998). This has important

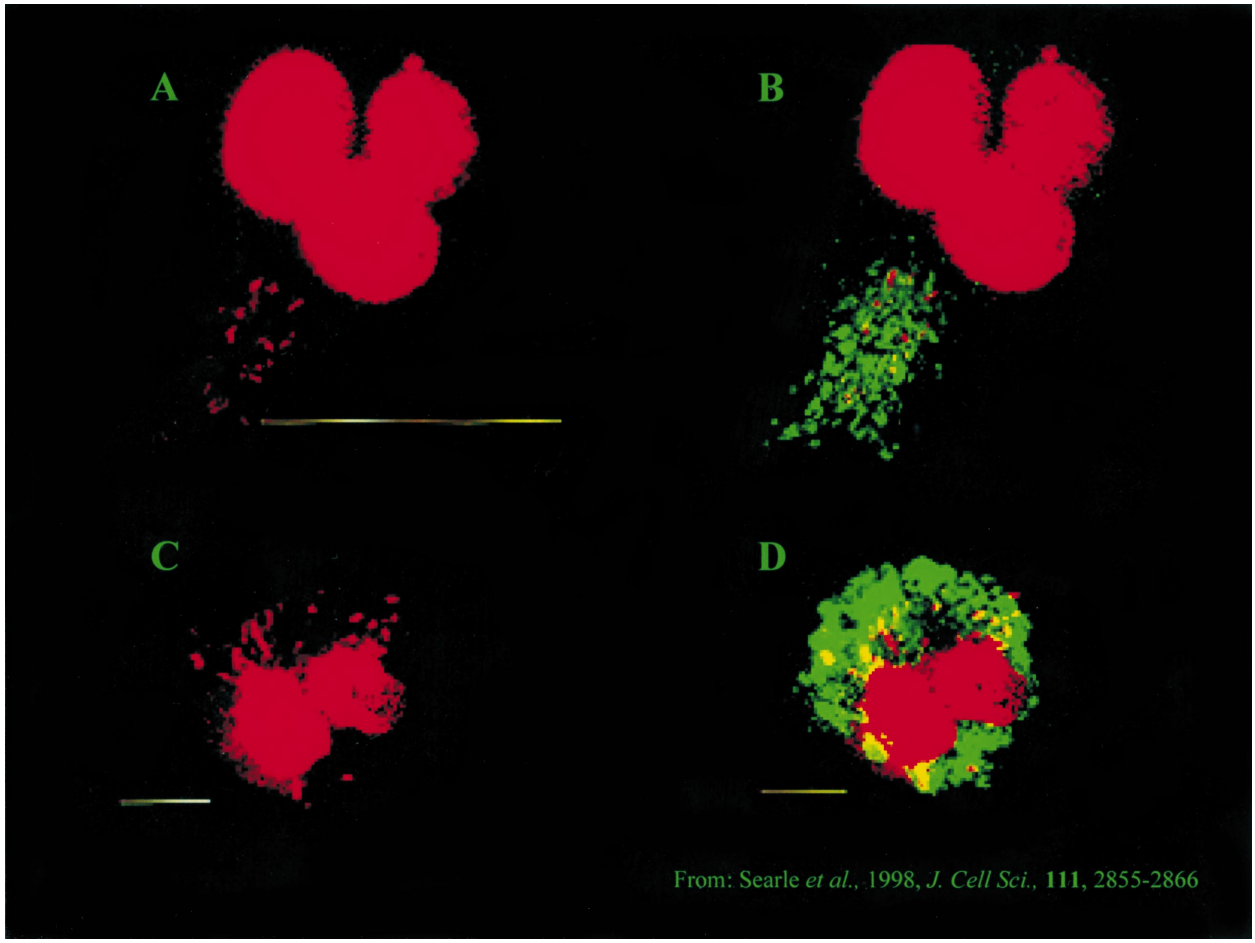

Fig. 1. Bone marrow-derived mϕ from Slc11a1 wild-type (A and B) versus mutant (C and D) congenic mice infected with *M. avium*.
A and C. Staining of mϕ nuclei and bacterial DNA with propidium iodide.
B and D. The same images merged with the green fluorescence channel showing anti-C-terminal anti-Slc11a1 staining. For wild-type mϕ (B), Slc11a1-positive vesicles have migrated to fuse with mycobacterial phagosomes. For mutant mϕ (D), no co-localization is observed. Reproduced with permission from Searle *et al.* (1998).

implications for any direct role that Slc11a1 might have in determining pathogen survival. For example, previous studies (Sturgill-Koszycki *et al.*, 1994; 1996) examining entry of *M. avium* and *M. tuberculosis* into bone marrow-derived mϕ from BALB/c (Slc11a1 mutant) mice indicated that maturation of the phagosome is arrested at an early transitional stage. The BALB/c phagosome acquires an immature form of cathepsin D from the TGN but remains accessible to internalized transferrin. Fusion with acidic endosomes or lysosomes and acquisition of proton-ATPases occurs only after activation with IFN-γ. Hence, it might be assumed that Slc11a1 would not have a direct influence on mycobacterial survival in resting macrophage, as the bacterium would not come into direct contact with the Slc11a1 protein. Instead, it seems that normal Slc11a1 function is required for maturation of the mycobacterial phagosome. This is consistent with reduced acidification of *Mycobacterium*-containing vesicles (Hackam *et al.*, 1998) and reduced phagosome–lysosome fusion (de Chastellier *et al.*, 1993; Hackam *et al.*, 1998) in *Mycobacterium*-infected mutant mϕ. In inactivated wild-type mϕ, normal acidification and phagosome–lysosome fusion occurs (de Chastellier *et al.*, 1993; Hackam *et al.*, 1998; Searle *et al.*, 1998), creating an environment in which Slc11a1 can directly influence antimicrobial activity (see below). These results suggest that the earlier observation (Sturgill-Koszycki *et al.*, 1996) of arrested phagosome development may be a feature peculiar to, or at least unnaturally prolonged in, Slc11a1 mutant mϕ. As Slc11a1 is also reduced in expression on young monocytes, this might also explain why Slc11a1 does not control visceralizing *L. major* infection that preferentially targets immature monocytes (Davies *et al.*, 1988), and may contribute to the failure of Slc11a1 to regulate *M. tuberculosis* infection in mice (Medina and North, 1996a,b; North *et al.*, 1999).

Slc11a2 localizes to early endosomes in mϕ (Gruenheid *et al.*, 1999) and other cell types throughout the body

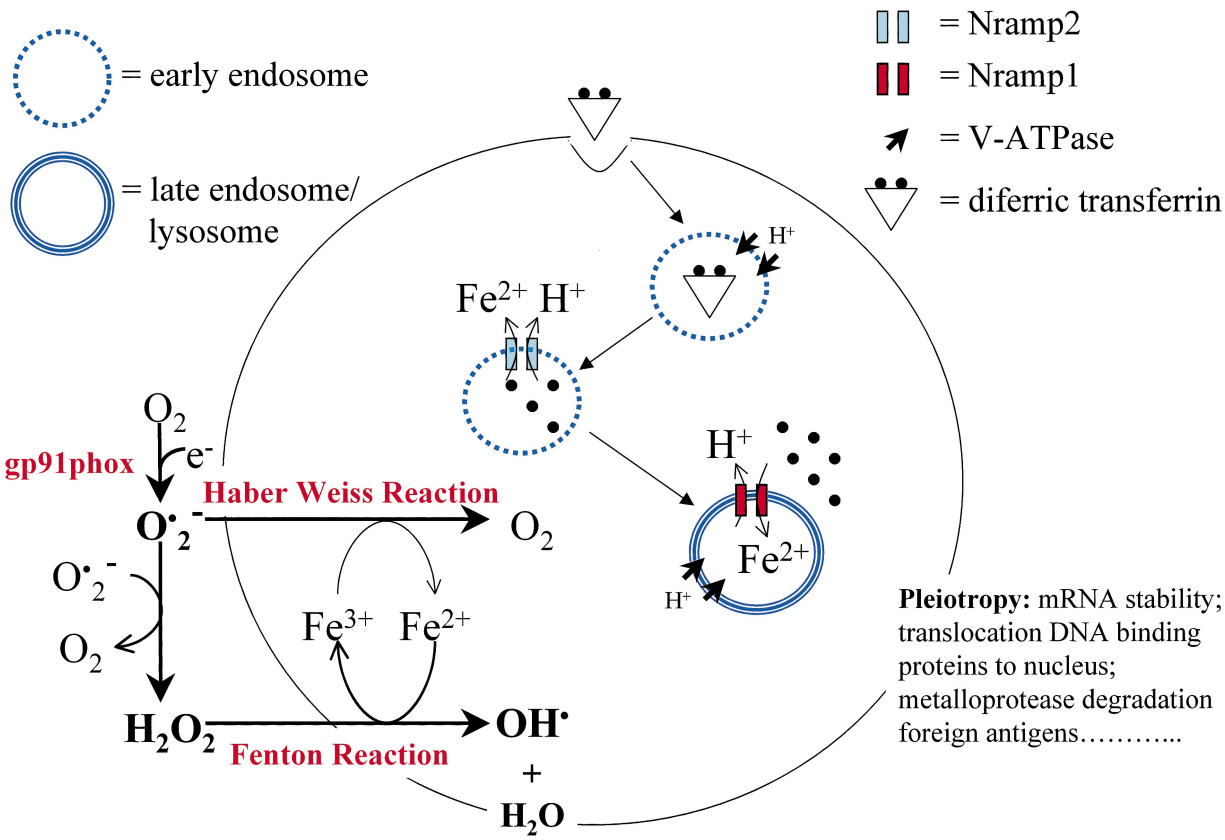

Fig. 2. Model of divalent cation homeostasis in mϕ and its relationship to oxygen- and nitrogen-dependent antimicrobial pathways. The symport activity of Slc11a2 delivers divalent cations (e.g. Fe^{2+}) to the cytosol across early endosomal membranes after recruitment of V-ATPase and acidification of the vacuole. The antiport activity of Slc11a1 delivers divalent cations from the cytosol to acidic late endosomes and lysosomes, where the Fenton reaction generates toxic antimicrobial radicals. Hydroxyl radicals ($OH^{\bullet}$) generated from the Fenton reaction may then react with nitric oxide (NO) to produce toxic peroxynitrate.

including the intestinal brush border (Fleming *et al.*, 1998; Canonne-Hergaux *et al.*, 1999), i.e. it appears to be expressed ubiquitously. Slc11a1 was thought to be restricted to the myeloid lineage (Vidal *et al.*, 1993), but we have recently localized it to vesicles in neurons (Evans *et al.*, 2001). This may relate directly to differences in behavioural response to stress (Evans *et al.*, 2001), activation of the hypothalamic–pituitary–adrenal axis (HPA) and baseline (Blackwell *et al.*, 1994) and post-stress (Evans *et al.*, 2001) mortality after *T. gondii* infection in mutant versus wild-type congenic mice. It also relates to the earlier observations of Zwilling and coworkers (Brown *et al.*, 1993) that activation of the HPA axis by restraint stress increased the severity of *M. avium* infection in *Slc11a1* mutant mice but did not affect the ability of congenic *Slc11a1* wild-type mice to control the mycobacterial infection. This was attributed to differences in the sensitivity of mϕ from *Slc11a1* congenic mice to corticosterone because HPA axis activation also caused increased intracellular growth of *M. avium* in mϕ from *Slc11a1* mutant but not wild-type mice (Brown and Zwilling, 1995; Brown *et al.*, 1995). These *in vivo* and *in vitro* differences were simulated by corticosterone administration and abrogated by surgical or pharmacological adrenalectomy. In our studies (Evans *et al.*, 2001), we observed enhanced levels of mRNA for corticotrophin-releasing hormone in the brain within 30 min of restraint stress. Hence, we hypothesized that, although differences in HPA activation translate into differences in adrenal enlargement and basal circulating corticosterone levels, the primary influence of Slc11a1 is at the level of the neuronal response to stress. These results highlight the importance of HPA activation in neuroimmune regulation of infectious disease and provide new insight into the possible roles of divalent cation transporters of the *Slc11a* gene family in regulating metal ion homeostasis in the brain and its broader pathological implications. For example, accumulation of metal ions in neurons is a feature of Parkinson's (Jellinger *et al.*, 1993; Gerlach *et al.*, 1994; Hirsch and Faucheux, 1998) and other (Gerlach *et al.*, 1996; Multhaup, 1997) neurodegenerative diseases. Intriguingly, the *Drosophila* orthologue *malvolio* also localizes to phagocytes and neurons and plays a role in taste behaviour (Rodrigues *et al.*, 1995), suggesting that this dual cellular localization may be a primitive feature of Slc11a1 expression.

Finding Slc11a1 in non-myeloid cells led us to reassess its expression profile. With both anti-N- and anti-C-terminal Slc11a1 antibodies, we confirmed Slc11a1 expression in cortical pyramidal neurons (principally layers III and V), a subset of striatal neurons, cerebellar Purkinje cell bodies and the anterior pituitary (N. Papo, J. M. Blackwell and J. K. White, unpublished). We also observed Slc11a1 in pancreatic islets and in the adrenal medulla. Expression in pancreatic islets is interesting because Slc11a1 is a candidate diabetes gene (Esposito *et al.*, 1998; Hill *et al.*, 2000).

Slc11a1 is a proton/divalent cation antiporter

Using *Xenopus* oocytes, we showed that, like Slc11a2 (Gunshin *et al.*, 1997), Slc11a1 is a divalent cation (Fe^{2+}, Zn^{2+} and Mn^{2+}) transporter (Goswami *et al.*, 2001). Strikingly, however, where Slc11a2 is a symporter of H^+ and metal ions, Slc11a1 is an antiporter that can flux divalent cations in either direction against a proton gradient. This provides a new model for metal ion homeostasis in mϕ (Fig. 2). Slc11a2 in early endosomes delivers extracellularly acquired divalent cations into the cytosol. Slc11a1 in late endosomes/lysosomes delivers divalent cations from the cytosol to this acidic compartment. Here, the Fenton reaction can use ferrous iron to generate toxic hydroxyl ($OH^{\bullet}$) radicals (Zwilling *et al.*, 1999). Mammalian cells, including mϕ, contain redox-active iron in lysosomes (Garner *et al.*, 1998), the exocytosis of which is important for oxidation and uptake of low-density lipoproteins (Yuan *et al.*, 1995). Monocyte-derived mϕ exposed to artificially aged erythrocytes show enhanced oxidation of low-density lipoproteins as a result of exocytosed iron (Yuan *et al.*, 1996).

That heavy metals accumulate in, and are exocytosed from, mϕ lysosomes was of interest in relation to the hypothesis (Atkinson and Barton, 1998; Fleming *et al.*, 1998) that Slc11a1 is involved in iron recycling from effete red cells by mϕ. With Jeremy Brock (University of Glasgow), we showed that iron phagocytosed via FcR as insoluble ^{59}Fe-labelled transferrin–antitransferrin immune complexes accumulates in mutant-transfected mϕ but is efficiently recycled to the medium in wild-type mϕ (Mulero *et al.*, submitted). This did not occur with ^{59}Fe-transferrin, which is taken up via recycling transferrin receptors, consistent with delivery to late endosomes/lysosomes as necessary for iron recycling by Slc11a1, possibly through lysosomal iron exocytosis. Interestingly, NO generation was essential to trigger iron release. The by-product of the high cytoplasmic iron in mutant mϕ is mRNA instability for a range of activation markers (Zwilling *et al.*, 1999; Lafuse *et al.*, 2000), contributing to the pleiotropy associated with Slc11a1 function. A role for Slc11a1 in recycling iron from effete red cells also implies a possible role in regulating iron homeostasis that would be interesting to examine in relation to the anaemias of chronic infection.

Controversy relating to Slc11a1 transport activity in mϕ

Our results using frog oocytes suggest that, depending on membrane topology (see below), antiport activity of Slc11a1 in mϕ will normally deliver divalent cations from the cytosol into acidic late endosomes/lysosomes. However, previous attempts to define Slc11a1 function in mϕ using radioisotopes and fluid-phase or particle-bound divalent cation-sensitive fluorescent probes provide contradictory results. Zwilling and colleagues showed that the rate of ^{55}Fe import by latex bead (Kuhn *et al.*, 1999) or *M. avium* (Zwilling *et al.*, 1999) phagosomes isolated from wild-type transfected mϕ was more than twice the rate observed in phagosomes from mutant mϕ. Phagosomes isolated from wild-type mϕ prelabelled with ^{55}Fe-citrate before phagocytosis also contained up to 4× Fe compared with mutant mϕ (Kuhn *et al.*, 1999). Using a divalent cation-sensitive fluorescent probe covalently attached to zymosan particles, other researchers (Jabado *et al.*, 2000) found that phagosomes from wild-type mϕ extrude Mn^{2+} faster than mutant mϕ, a difference eliminated by preventing phagosomal acidification. This was shown by measuring the rate of quenching of particle-bound fluorescence after the addition of exogenous 500 μM Mn^{2+}. Interestingly, the ability (Zwilling *et al.*, 1999) to inhibit mycobacterial growth in wild-type mϕ by the addition of exogenous iron was dose dependent, reaching a maximum at 0.05 μM iron, decreasing and eventually lost at higher concentrations up to 0.5 μM, suggesting some change in Slc11a1 function at higher exogenous iron concentration. Our data demonstrate that Slc11a1 can flux divalent cations in either direction depending upon the pH on either side of the membrane. Net flux will be determined by the combined electrochemical gradients of the metal and H^+, which may explain the discrepancies in results from different laboratories using different techniques and experimental conditions to evaluate the direction of transport across mϕ phagosomal membranes. More refined studies in mϕ are required to determine the conditions under which influx versus efflux of divalent cations from Slc11a1-positive vesicles occurs. It is possible that, as in yeast, late endosomes/lysosomes of mϕ act as 'storage vacuoles' for divalent cations that can be fluxed in or out according to varying conditions of metal ion stress or depletion in the cytosol.

Slc11a1 pleiotropy and infectious disease susceptibility

One effect of Slc11a1 is enhanced expression of iNOS

(encoded by *Nos2A*) and generation of toxic NO in wild-type versus mutant mϕ (Roach *et al*., 1991; Arias *et al*., 1997). It was assumed that this is crucial to Slc11a1-mediated resistance *in vivo*, perhaps combining with $OH^{\bullet}$ to produce the more toxic peroxynitrite. Using immunofluorescence, we examined (C. Evans and J. M. Blackwell, unpublished) Slc11a1 and iNOS expression and localization in the liver early in *L. donovani* infection in *Slc11a1* congenic mice. Slc11a1 is expressed in iNOS-negative resident Kupffer cells but not in iNOS-positive:-Mac1-positive fresh monocytes entering the liver. In wild-type mice, monocytes (now mϕ) within developing granulomas become Slc11a1 positive at ≈10 days of infection. In mutant mice, they do not. This suggests that iNOS is not important in the early Slc11a1-mediated Kupffer cell regulation (Crocker *et al*., 1984) of infection, but may play a role later in granuloma-dependent (Stern *et al*., 1988) control of the parasite. To evaluate further the role of iNOS, we have backcrossed the *Nos2A* knock-out (KO) (Wei *et al*., 1995) onto *Slc11a1* wild-type and mutant congenic mice on C57BL/10ScSn (= B10), C57BL/6 (= B6) and BALB backgrounds. This will allow us to determine the importance of iNOS in Slc11a1-regulated *L. donovani*, *S. typhimurium* and *M. avium* infections. We are also intercrossing the B6 gp91phox KO (Shiloh *et al*., 1999) with our B6 *Slc11a1*/*Nos2A* KO congenics. Phenotypic analyses will allow us to determine whether NO and O_2^- contribute separately or syngergistically in Slc11a1-regulated infectious and autoimmune disease phenotypes. The possible role of gp91phox in radical generation becomes more interesting given its contribution of O_2^- as substrate for superoxide dismutase (SOD)-generated H_2O_2, which in turn acts as a substrate for Fenton reaction reduction of Fe^{2+}, the latter contributing its electron to $OH^{\bullet}$ (Fig. 2). The reductase gp91phox is crucial for the generation of O_2^- from O_2, but may also act directly as a ferrireductase (C. M. Proctor and N. Robinson, personal communication). Gp91phox is delivered to infected phagosomes by vesicular fusion (Vazquez-Torres *et al*., 2000), and we are evaluating this using anti-gp91phox antibody and confocal microscopy. Whether its role is solely to generate antimicrobial O_2^- and/or to act as a ferrireductase to provide ferrous iron for Slc11a1 Fe^{2+} influx or efflux is not known. Further intercrosses, e.g. crossing in a SOD KO, will tease out the role of gp91phox. Other intercrosses, e.g. knock-outs for TNFα, IL-1β, chemokines or their receptors, will determine the roles of other proinflammatory molecules in Slc11a1-regulated phenotypes.

In other studies, we showed that Slc11a1 wild-type mϕ have enhanced lipopolysaccharide-dependent antigen processing for presentation to T cells (Lang *et al*., 1997), which may reflect metal ion requirement for metalloprotease activity and/or endosomal fusion events. This effect on antigen processing is compounded by Slc11a1's influence on molecules regulating (TNFα, IL-1β) or directly involved in (MHC class II) antigen presentation. Hence, in mice vaccinated with attenuated *S. typhimurium* engineered to express tetanus toxoid fragment C, we found polarized T helper 1 (Th1) versus T helper 2 (Th2) responses in congenic wild-type versus mutant mice (Soo *et al*., 1998). This Th1:Th2 bias was also seen by us in *L. donovani* infection (Kaye and Blackwell, 1989) and by others in mycobacterial infection (Kramnik *et al*., 1994).

One of the perplexities in SLC11A1 research is the clear association between both 5′ and 3′ haplotypes and global susceptibility to pulmonary tuberculosis in man (Shaw *et al*., 1997; Bellamy *et al*., 1998; Cervino *et al*., 2000; Gao *et al*., 2000; Greenwood *et al*., 2000; Ryu *et al*., 2000), but the lack of any effect of Slc11a1 on primary infection with *M. tuberculosis* (Medina and North, 1996a,b; North *et al*., 1999) in mice. One clear difference is that humans, especially in Africa, are repeatedly exposed to environmental mycobacteria before infection with *M. tuberculosis*. In Malawi, we showed (J. M. Blackwell, S. Floyd, G. Black, H. Dockrell and P. E. M. Fine, unpublished) significant SLC11A1 haplotype associations with IFN-γ responses to *M. tuberculosis and Mycobacterium fortuitum* antigens, consistent with the murine Th1:Th2 bias. Our results suggest that polymorphism at SLC11A1 influences immune response to 'priming/vaccinating' exposures to mycobacteria. This hypothesis is interesting, in that BCG's effectiveness in protecting against leprosy in the same Malawian population is also associated with polymorphism at SLC11A1 (A. V. Hill and P. E. M. Fine, personal communication). No SLC11A1 association with leprosy is observed in non-BCG-vaccinated subjects. Polymorphism at SLC11A1 is also associated with Mitsuda-type skin test reactivity to leprosy antigens (Alcais *et al*., 2000). These results are also interesting in relation to recent studies suggesting that the adoption of a Western infectious disease-free lifestyle is associated with an increase in the prevalence of allergic and autoimmune diseases, which can also be modulated by immune response to mycobacterial antigens after BCG vaccination (Shirakawa *et al*., 1997). Stratification by BCG vaccination was recently shown by us to unmask a genetic risk factor for atopy in the region of the SLC11A1 locus (Alm *et al*., 2001), pointing to the importance of genotype by environment interactions in determining disease susceptibility.

Functional polymorphism at human SLC11A1

Using transient transfection and a luciferase reporter gene, we have shown (Searle and Blackwell, 1999) that a repeat polymorphism, designated $(GT)_n$, identified by us

(Blackwell *et al.*, 1995) in the promoter of human SLC11A1 regulates expression. The sequence contains a Z-DNA repeat with four alleles: (i) $t(gt)_5ac(gt)_5ac(gt)_{11}g$; (ii) $t(gt)_5ac(gt)_5ac(gt)_{10}g$; (iii) $t(gt)_5ac(gt)_5ac(gt)_9g$; (iv) $t(gt)_5ac(gt)_9g$. Alleles (i) and (iv) are rare (gene frequencies ≈ 0.001); alleles (ii) and (iii) occur at gene frequencies of ≈ 0.25 and ≈ 0.75 respectively. In the absence of exogenous stimuli, allele (iii) drives five- to eightfold higher reporter gene expression than alleles (i), (ii) and (iv). All alleles show similar percentage enhancement of expression with IFN-γ, consistent with multiple IFN-γ response elements 5′ and 3′ of the repeat. The addition of lipopolysaccharide has no effect on alleles (i) and (iv), but causes significant reduction in expression driven by allele (ii) and enhances expression driven by allele (iii). Juxtaposition of lipopolysaccharide-related response elements (NFκB, AP-1, NF-IL6) may thus be differentially affected by the two common alleles.

Disease associations with SLC11A1

The multiple pleiotropic effects on mϕ activation prompted us to look for human SLC11A1 association with autoimmune as well as infectious disease (Shaw *et al.*, 1996). This has since been replicated in multiple studies (Hofmeister *et al.*, 1997; Esposito *et al.*, 1998; Maliarik *et al.*, 2000; Sanjeevi *et al.*, 2000; Singal *et al.*, 2000; Yang *et al.*, 2000). We also proposed that the high expressing allele (iii) of the $(GT)_n$ polymorphism would be associated with autoimmune disease, and the low expressing allele (ii) with infection. This too has held true across multiple autoimmune (Shaw *et al.*, 1996; Esposito *et al.*, 1998; Sanjeevi *et al.*, 2000) and infectious (Shaw *et al.*, 1997; Bellamy *et al.*, 1998; Gao *et al.*, 2000) disease (specifically tuberculosis) studies, suggesting that there may be some balancing selective forces that keep both alleles in the population. In this respect, it is interesting that allele frequencies for SLC11A1 allele (ii) are in the range 0.14–0.20 throughout western and southern Africa (0.16 South African Cape coloureds; 0.20 Malawi; 0.14 The Gambia) compared with 0.25–0.29 in northern European (0.25 UK; 0.27 Sweden; 0.29 Latvia) populations and 0.36 in Brazil. Recently, we showed (M. Hibbard, M. Levin and J. M. Blackwell, unpublished) that homozygous 3/3 individuals produce higher levels of TNFα and are at significantly higher risk of severe clinical meningococcal disease. This is again consistent with high SLC11A1 expression generating proinflammatory responses during acute bacteraemia. Intriguingly, allele (iii) is also on a 5′ haplotype significantly associated with visceral leishmaniasis and post-kala-azar dermal leishmaniasis in Sudan (Mohamed *et al.*, submitted), consistent with SLC11A1-regulated proinflammatory responses (e.g. high TNFα; Barral-Netto *et al.*, 1991) associated with this disease and with allele (ii) being at a higher frequency (0.27) in this population. Hence, a pathogenic organism's ability or not to elicit an acute proinflammatory response may also contribute to the selective forces influencing the maintenance of SLC11A1 alleles in the population. In many studies, evidence has also been found for polymorphisms on 3′ haplotypes contributing separately to disease susceptibility, suggesting that there are other functional mutations yet to be identified in man. Clearly, more detailed population genetic analyses will be necessary to understand the relationship between more complex haplotypes and disease incidence in different geographic regions. We are currently sequencing across the SLC11A1 to identify new functional coding and/or regulatory polymorphisms contributing to disease susceptibility in different populations.

Evolution of the Nramp gene family

Following our demonstration that Slc11a1 and Slc11a2 have different modes of action, we were interested in the evolution of Nramp genes. In yeast, Nramp homologues include *SMF1/2/3*. Smf1p/2p localize to the outer membrane and are involved in Cu^{2+}, high-affinity Mn^{2+} and, possibly, Fe^{2+} transport into the cell (Supeck *et al.*, 1996; Cohen *et al.*, 2000). Smf1p and Smf2p localize to distinct cellular compartments under metal starvation (Portnoy *et al.*, 2000): Smf1p accumulates at the cell surface; Smf2p is restricted to intracellular vesicles. Smf3p is quite distinctive. It is downregulated by iron and localizes to vacuolar membranes independently of metal treatment. Yeast lacking Smf3p show symptoms of iron starvation, suggesting that Smf3p helps to mobilize (i.e. efflux) iron from vacuolar stores. Murine Slc11a2 but not Slc11a1 complements the Mn^{2+} transport and pH-sensitive phenotype in *smf1/2* double KO *Saccharomyces cerevisiae* (Pinner *et al.*, 1997). This could be explained by differential localization (e.g. membrane versus vacuole) in yeast, or symport versus antiport functions. Similarly, human SLC11A2 but not SLC11A1 complements the EGTA- and pH-sensitive phenotype of the divalent metal transporter-deficient pdt1Delta strain of *Schizosaccharomyces pombe* (Tabuchi *et al.*, 1999). Here, replacement of the N-terminus of SLC11A2 with that of SLC11A1 results in an inactive chimera, indicating that the N-termini of mammalian Nramps differentially regulate function.

To try to determine when mammalian Nramps diverged in localization and function, two orthologues (*fSlc11*α/*fSlc11*β) were sequenced by us (Sibthorpe *et al.*, submitted) from the puffer fish *Fugu rubripes*. Phylogenetic analysis shows that both are similar to mammalian Slc11a2, suggesting that Slc11a2 is ancestral. The gene environment for both is syntenic with mouse chromosome 15, consistent with gene duplication in the ancestral Slc11a2 location. Only *fSlc11*α has an N-terminal tyrosine-based putative endosomal targeting signal and rudiments of a

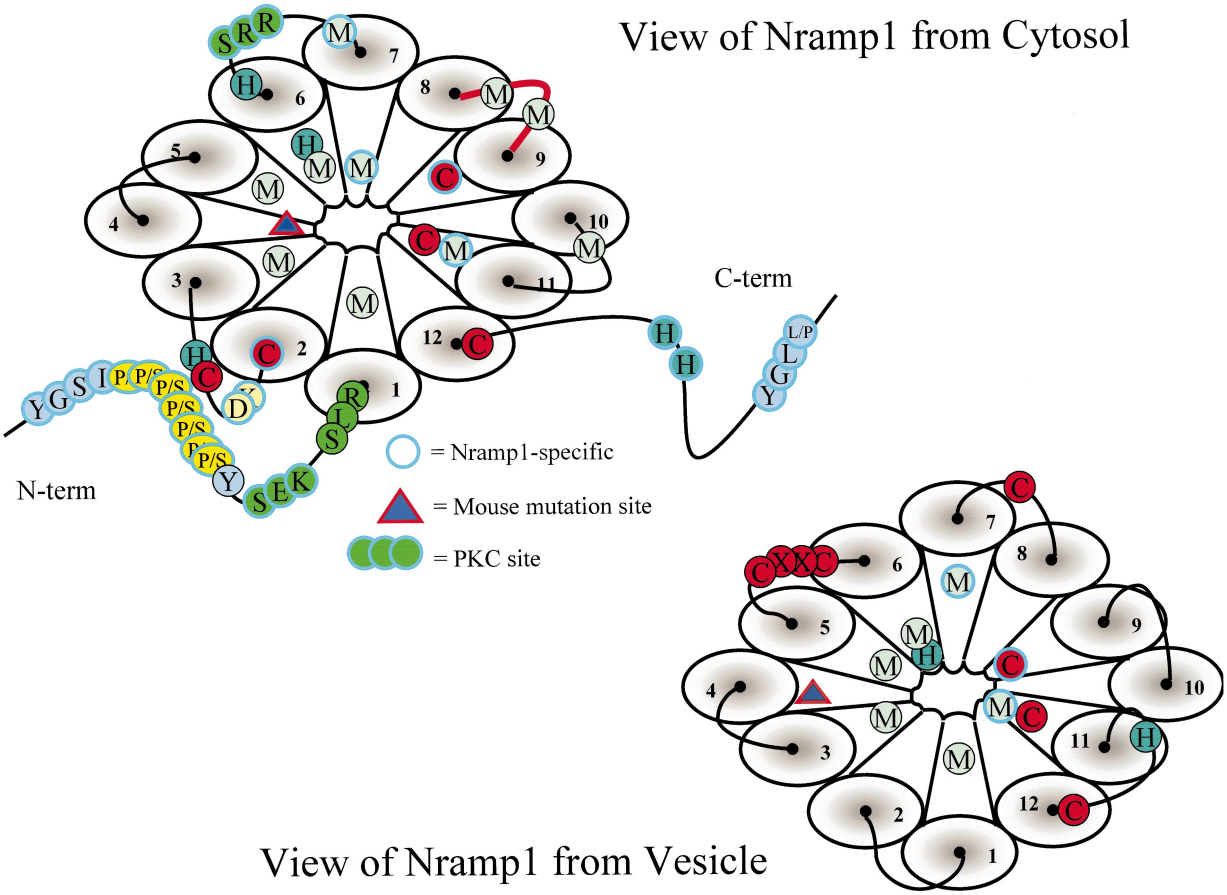

Fig. 3. Slc11a1 as viewed from the cytosol or the vesicle assuming a topology in which N- and C-termini are cytosolic. This diagrammatic representation shows the locations of: conserved and Slc11a1-specific Cys (C), His (H) and Met (M) residues; N-and C-terminal YXXZ putative endosomal targeting motifs (where X = any amino acid, Z = bulky, hydrophobic residue); the Pro–Ser-rich N-terminus; PKC binding sites; and the position of the murine transmembrane domain 4 functional null mutation.

Pro–Ser-rich N-terminal sequence similar to Slc11a1. Transient transfection of green fluorescent protein-tagged constructs into human epithelial kidney cells confirms a late endosomal/lysosomal localization for *fSlc11α*, consistent with divergence towards an Slc11a1-like function. Further work is required to see whether *fSlc11α* is an antiporter. Interestingly, human SLC11A1 complements *Drosophila malvolio*, suggesting that Slc11a1-like antiport activity emerged earlier in Nramp evolution. Two functional classes of NRAMP proteins also occur in plants: *Arabidopsis thaliana* (At) SLC11A1 and *Oriza sativa* (Os) SLC11A1 and 3 represent one class; AtSLC11A2–5 and OsSLC11A2 the other (Curie *et al.*, 2000). AtSlc11a1 and OsSlc11a1 complement the *fet3fet4* yeast mutant defective in both low- and high-affinity iron transports, whereas AtSlc11a2 and OsSlc11a2 fail to do so.

Nramps at the host–pathogen interface

The Nramp gene family is highly conserved across prokaryotes and eukaryotes, with orthologues now identified in pathogenic bacteria that come under Slc11a1 control in mϕ (Agranoff *et al.*, 1999; Kehres *et al.*, 2000). This means that the pathogen's own requirement for transition metal ions and genetic variation at its own Nramp-related proteins may be influenced by mammalian Nramp polymorphism and function, providing a dynamic interface upon which the forces of evolution have been acting.

Role of other divalent cation transporters in disease

Many molecules mediate metal ion homeostasis, including metallochaperones, ferritins, metallothionines and regulatory proteins that adjust the expression or function of metal trafficking transporters. A dysfunction anywhere in the metal-sensing pathway may cause disease. However, the two copper transporters associated with Menkes and Wilson's diseases (reviewed by Harris, 2000) provide particularly intriguing molecular and cell biological parallels with Slc11a functions. For both, yeast complementation

has been used successfully to detect defects in copper transport, and mammalian cell assays to identify defects in intracellular trafficking. The Menkes protein (ATP7A) is a P-type ATPase defective in X-linked recessive copper deficiency, leading to decreased copper in the brain, neurodegeneration and early death. More than 150 mutations occur in man. ATP7A normally localizes to the trans-Golgi network (TGN) and constitutively cycles via the plasma membrane in basal copper conditions (Petris and Mercer, 1999). Under copper stress, ATP7A is recruited preferentially to the plasma membrane where it effluxes copper. Endocytosis of ATP7A under both basal and elevated copper is mediated by a C-terminal dileucine motif (Francis *et al*., 1999; Petris and Mercer, 1999), but other, including transmembrane domain-located, signals are involved in TGN retention and retrieval from endosomes to the TGN (Ambrosini and Mercer, 1999). ATP7A has eight transmembrane domains and a long N-terminus with six copper binding sites (GMxCxxC). These are involved in copper sensing, provide signals for relocalization from the TGN to the plasma membrane (Strausak *et al*., 1999) and chaperone copper to the centre of the channel, although some copper can be delivered to the channel directly (Voskoboinik *et al*., 1999). Copper also binds to Cys, Met and His residues on transmembrane domains, forming a transient complex during transport. Mutations in patients influence copper binding, localization, trafficking or transport functions. In autosomal Wilson's disease, more than 200 mutations in *ATP7B* influence localization and transport function and are associated with high levels of tissue copper, particularly in the liver, brain and kidney. ATP7B localizes to the TGN and late endosomes (Harada *et al*., 2000) under basal conditions, with high copper influencing redistribution to vesicles and to apical vacuoles/membranes in polarized cells. In this case, copper binding to the N-terminal domain is associated with conformational change that leads to weakening of the interaction between the ATP-binding domain/loop and the N-terminal domain (Vanderwerf *et al*., 2001). Alteration in domain–domain interactions is coupled to changes in the nucleotide-binding properties of the ATP-binding domain, suggesting that copper binding to the N-terminus modulates transport activity. Increase in copper leads to hyperphosphorylation of ATP7B, and the level of phosphorylation correlates with intracellular localization. Regulation via phosphorylation requires the presence in the protein of the properly folded N-terminal domain.

Using knowledge of ATP7A and ATP7B to relate Slc11a1 structure to function

At present, we know very little about how Slc11a1/SLC11A1 protein sequence and structure relates to function. Figure 3 provides a diagrammatic representation of how Slc11a1 might lie in the late endosomal/lysosomal membrane, highlighting key features of the molecule that may be important to function. Several key questions remain to be addressed.

Question 1 is to determine the topology of Slc11a1 and Slc11a2 in early endosome versus late endosome/lysosome vesicular membranes. Logic dictates that both will have N- and C-termini and conserved transport motif intertransmembrane loop 8 on the cytoplasmic face, with intertransmembrane loops 1, 3, 5, 7, 9 and 11 facing into the vesicle. Symport versus antiport activity then determines that divalent cation flows from vesicle to cytosol for Slc11a2-positive early endosomes and from cytosol to vesicle when Slc11a1-positive late endosomes/lysosomes are acidic. However, Kuhn *et al*. (2001) have reported that antibody to intertransmembrane loop 7 inhibited divalent cation uptake into isolated mϕ phagosomes, whereas an anti-C-terminal antibody did not. Although the latter may be because the antibody did not provide the correct steric hindrance, the former could only be accounted for by the antibody accessing the intravesicular compartment under the 'logical' model. Paradoxically, Kuhn *et al*. (2001) also report that inhibitors of PKC diminished iron import to phagosomes. This would be consistent with PKC access to binding sites on Slc11a1, all of which lie on the same face as N- and C-termini. Unless PKC could, in this case, gain intravesicular access, there is internal inconsistency in the data. To determine topology, we are currently analysing fluorescence in selectively permeabilized cells after transient transfection of intertransmembrane loop FLAG-tagged Slc11a1 constructs.

Question 2 is to determine when symport versus antiport activity diverged in the Nramp gene family. In our laboratory, we are approaching this in two ways: (i) allowing evolution to inform us of divergence in function by testing *fSlc11*α/β in oocyte functional screens; and (ii) testing chimeric Slc11a1/Slc11a2 constructs to identify regions that determine antiport versus symport function. We can then pinpoint conserved sequences in these regions that might inform us further as to the molecular evolution of divergent functions.

Question 3 is to ascertain the key amino acid and motifs that determine the range and specificity of Slc11a1 divalent cation transport. By analogy with other metal ion-binding and transport proteins, conserved Cys, His and Met residues are likely to be important. Figure 3 demonstrates the potential juxtaposition of conserved residues around a putative pore formed by the 12 transmembrane domains in the vesicular membrane. Conserved residues, and a conserved CxxC motif, also occur on intertransmembrane loops on both faces of the molecule. Five Slc11a1-specific Cys, His and Met residues are conserved across mammalian and chicken

Slc11a1. We are currently testing transport function and divalent cation specificity using constructs with targeted mutation in conserved Slc11a1/Slc11a2 and Slc11a1-specific, residues.

Question 4 relates to changes in Slc11a1 function with mϕ activation and the potential importance of PKC binding sites and the N-terminal Pro–Ser-rich domain. PKC activity has been implicated in regulating iron uptake by phagosomes (Kuhn *et al*., 2001). Using mutated constructs, we are looking at effects on protein phosphorylation, determining which PKC sites affect transport function, and using glutathione-*S*-transferase pull-down assays to look for interactions between the N- and/or C-terminal domains and putative cytosolic loops of Slc11a1.

Furthering our understanding of the role of SLC11A1 in disease

Transition metal ions are essential for life and participate in many cellular functions. These include regulation of transcription through DNA-binding proteins and metal response elements, the functions of hundreds of enzymes, including metalloproteases, SOD and iNOS, and cellular functions such as endosomal fusion (Aballay *et al*., 1995). The fact that Slc11a1 transports Fe^{2+}, Zn^{2+} and Mn^{2+} and possibly other divalent cations provides the crucial clue to pleiotropy. This reflects not only direct regulation of vesicular divalent cation content, but also secondary effects on concentrations in the cytosol and surrounding cellular milieu. The challenge for the future is to determine precisely which of the direct or indirect pleiotropic effects of SLC11A1 are most important in determining each of the multiple disease associations now observed in man.

Acknowledgements

Our work on Nramps is supported by The Wellcome Trust

References

Aballay, A., Sarrouf, M.N., Colombo, M.I., Stahl, P.D., and Mayorga, L.S. (1995) Zn2+ depletion blocks endosome fusion. *Biochem J* **312**: 919–923.

Abel, L., Sanchez, F.O., Oberti, J., Thuc, N.V., Hoa, L.V., Lap, V.D., *et al.* (1998) Susceptibility to leprosy is linked to the human NRAMP1 gene. *J Infect Dis* **177**: 133–145.

Agranoff, D., Monahan, I.M., Mangan, J.A., Butcher, P.D., and Krishna, S. (1999) *Mycobacterium tuberculosis* expresses a novel pH-dependent divalent cation transporter belonging to the Nramp family. *J Exp Med* **190**: 717–724.

Alcais, A., Sanchez, F.O., Thuc, N.V., Lap, V.D., Oberti, J., Lagrange, P.H., *et al.* (2000) Granulomatous reaction to intradermal injection of lepromin (Mitsuda reaction) is linked to the human NRAMP1 gene in Vietnamese leprosy sibships. *J Infect Dis* **181**: 302–308.

Alm, J.S., Sanjeevi, C.B., Miller, E.N., Dabadghao, P., Lilja, G., Pershagen, G., *et al.* (2001) Atopy in children in relation to BCG vaccination and genetic polymorphisms at SLC11A1 (formerly NRAMP1) and D2S1471. *Genes Immun* (in press)

Ambrosini, L., and Mercer, J.F. (1999) Defective copper-induced trafficking and localization of the Menkes protein in patients with mild and copper-treated classical Menkes disease. *Hum Mol Genet* **8**: 1547–1555.

Arias, M., Rojas, M., Zabaleta, J., Rodriguez, J.I., Paris, S.C., Barrera, L.F., and Garcia, L.F. (1997) Inhibition of virulent *Mycobacterium tuberculosis* by Bcg(r) and Bcg(s) macrophages correlates with nitric oxide production. *J Infect Dis* **176**: 1552–1558.

Atkinson, P.G.P., and Barton, C.H. (1998) Ectopic expression of Nramp1 in COS-1 cells modulates iron accumulation. *FEBS Lett* **425**: 239–242.

Atkinson, P.G.P., Blackwell, J.M., and Barton, C.H. (1997) Nramp1 locus encodes a 65 kDa interferon-g inducible protein in murine macrophages. *Biochem J* **325**: 779–786.

Barral-Netto, M., Badaro, R., Barral, A., Almeida, R.P., Santos, S.B., Badaro, F., *et al.* (1991) Tumor necrosis factor (cachectin) in human visceral leishmaniasis. *J Infect Dis* **163**: 853–857.

Barton, C.H., White, J.K., Roach, T.I.A., and Blackwell. J.M. (1994) NH2-terminal sequence of macrophage-expressed natural resistance-associated macrophage protein (Nramp) encodes a proline/serine-rich putative Src homology 3-binding domain. *J Exp Med* **179**: 1683–1687.

Bellamy, R., Ruwende, C., Corrah, T., McAdam, K.P.W.J., Whittle, H.C., and Hill, A.V.S. (1998) Variation in the NRAMP1 gene is associated with susceptibility to tuberculosis in West Africans. *N Engl J Med* **338**: 640–644.

Blackwell, J.M., and Searle, S. (1999) Genetic regulation of macrophage activation: understanding the function of Nramp1 (= Ity/Lsh/Bcg). *Immunol Lett* **65**: 73–80.

Blackwell, J.M., Roberts, C.W., Roach, T.I.A., and Alexander, J. (1994) Influence of macrophage resistance gene Lsh/Ity/Bcg (candidate Nramp) on *Toxoplasma gondii* infection in mice. *Clin Exp Immunol* **97**: 107–112.

Blackwell, J.M., Barton, C.H., White, J.K., Searle, S., Baker, A.-M., Williams, H., and Shaw, M.-A. (1995) Genomic organization and sequence of the human NRAMP gene: identification and mapping of a promoter region polymorphism. *Mol Med* **1**: 194–205.

Blackwell, J.M., Searle, S., Goswami, T., and Miller, E.N. (2000) Understanding the multiple functions of Nramp1. *Microbes Infect* **2**: 317–321.

Brown, D.H., and Zwilling, B.S. (1994) Activation of the hypothalamic-pituitary-adrenal axis differentially affects the anti-mycobacterial activity of macrophages from BCG-resistant and susceptible mice. *J Neuroimmunology* **53**: 181–187.

Brown, D.H., Sheridan, J., Pearl, D., and Zwilling, B.S. (1993) Regulation of mycobacterial growth by the hypothalamus-pituitary-adrenal axis: differential responses of *Mycobacterium bovis* BCG-resistant and -susceptible mice. *Infect Immun* **61**: 4793–4800.

Brown, D.H., LaFuse, W., and Zwilling, B.S. (1995) Cytokine-mediated activation of macrophages from *Mycobacterium bovis* BCG-resistant and -susceptible mice: differential effects of corticosterone on antimycobacterial activity and expression of the *Bcg* gene (candidate *Nramp*). *Infect Immun* **63**: 2983–2988.

Brown, I.N., Glynn, A.A., and Plant, J. (1982) Inbred mouse strain

resistance to *Mycobacterium lepraemurium* follows the Ity/Lsh pattern. *Immunology* **47**: 149–156.

Canonne-Hergaux, F., Gruenheid, S., Ponka, P., and Gros, P. (1999) Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. *Blood* **93**: 4406–4417.

Cervino, A.C., Lakiss, S., Sow, O., and Hill, A.V. (2000) Allelic association between the NRAMP1 gene and susceptibility to tuberculosis in Guinea-Conakry. *Ann Hum Genet* **64**: 507–512.

de Chastellier, C., Frehel, C., Offredo, C., and Skamene, E. (1993) Implication of phagosome-lysosome fusion in restriction of *Mycobacterium avium* growth in bone marrow macrophages from genetically resistant mice. *Infect Immun* **61**: 3775–3784.

Cohen, A., Nelson, H., and Nelson, N. (2000) The family of SMF metal ion transporters in yeast cells. *J Biol Chem* **275**: 33388–33394.

Crocker, P.R., Blackwell, J.M., and Bradley, D.J. (1984) Expression of the natural resistance gene Lsh in resident liver macrophages. *Infect Immun* **43**: 1033–1040.

Curie, C., Alonso, J.M., Le Jean, M., Ecker, J.R., and Briat, J.F. (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochem J* **347**: 749–755.

Davies, E.V., Singleton, A.M., and Blackwell. J.M. (1988) Differences in *Lsh* gene control over systemic *Leishmania major* and *Leishmania donovani* or *Leishmania mexicana mexicana* infections are caused by differential targeting to infiltrating and resident liver macrophage populations. *Infect Immun* **56**: 1128–1134.

Esposito, L., Hill, N.J., Pritchard, L.E., Cucca, F., Muxworthy, C., Merriman, M.E., *et al.* (1998) Genetic analysis of chromosome 2 in type 1 diabetes: analysis of putative loci IDDM7, IDDM12, and IDDM13 and candidate genes NRAMP1 and IA-2 and the interleukin-1 gene cluster. IMDIAB Group. *Diabetes* **47**: 1797–1799.

Evans, C.A.W., Harbuz, M.S., Ostenfeld, T., Norrish, A., and Blackwell, J.M. (2001) Nramp1 is expressed in neurons and is associated with behavioural and immune responses to stress. *Neurogenetics* **3**: 69–78.

Fleming, M.D., Trenor, C.C., Su, M.A., Foernzler, D., Beier, D.R., Dietrich, W.F., and Andrews, N.C. (1997) Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature Genet* **16**: 383–386.

Fleming, M.D., Romano, M.A., Su, M.A., Garrick, L.M., Garrick, M.D., and Andrews, N.C. (1998) Nramp2 is mutated in the anemic Belgrade (b) rat: Evidence of a role for Nramp2 in endosomal iron transport. *Proc Natl Acad Sci USA* **95**: 1148–1153.

Francis, M.J., Jones, E.E., Levy, E.R., Martin, R.L., Ponnambalam, S., and Monaco, A.P. (1999) Identification of a di-leucine motif within the C terminus domain of the Menkes disease protein that mediates endocytosis from the plasma membrane. *J Cell Sci* **112**: 1721–1732.

Gao, P.S., Fujishima, S., Mao, X.Q., Remus, N., Kanda, M., Enomoto, T., *et al.* (2000) Genetic variants of NRAMP1 and active tuberculosis in Japanese populations. International Tuberculosis Genetics Team. *Clin Genet* **58**: 74–76.

Garner, B., Roberg, K., and Brunk, U.T. (1998) Endogenous ferritin protects cells with iron-laden lysosomes against oxidative stress. *Free Radic Res* **29**: 103–114.

Gerlach, M., Ben-Shachar, D., Riederer, P., and Youdim, M.B. (1994) Altered brain metabolism of iron as a cause of neurodegenerative diseases? *J Neurochem* **63**: 793–807.

Gerlach, M., Riederer, P., and Youdim, M.B. (1996) Molecular mechanisms for neurodegeneration. Synergism between reactive oxygen species, calcium, and excitotoxic amino acids. *Adv Neurol* **69**: 177–194.

Goswami, T., Bhattacharjee, A., Babal, P., Searle, S., Moore, E., Li, M., and Blackwell, J.M. (2001) Natural-resistance-associated macrophage protein 1 is an H+/bivalent cation antiporter. *Biochem J* **354**: 511–519.

Goto, Y., Buschman, E., and Skamene, E. (1989) Regulation of host resistance to *Mycobacterium intracellulare in vivo* and *in vitro* by the Bcg gene. *Immunogenetics* **30**: 218–221.

Greenwood, C.M., Fujiwara, T.M., Boothroyd, L.J., Miller, M.A., Frappier, D., Fanning, E.A., *et al.* (2000) Linkage of tuberculosis to chromosome 2q35 loci, including NRAMP1, in a large aboriginal Canadian family. *Am J Hum Genet* **67**: 405–416.

Gruenheid, S., Cellier, M., Vidal, S., and Gros, P. (1995) Identification and characterization of a second mouse Nramp gene. *Genomics* **25**: 514–525.

Gruenheid, S., Pinner, E., Desjardins, M., and Gros, P. (1997) Natural resistance to infections with intracellular pathogens: The Nramp1 protein is recruited to the membrane of the phagosome. *J Exp Med* **185**: 717–730.

Gruenheid, S., Canonne-Hergaux, F., Gauthier, S., Hackam, D.J., Grinstein, S., and Gros, P. (1999) The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. *J Exp Med* **189**: 831–841.

Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., *et al.* (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388**: 482–488.

Hackam, D.J., Rotstein, O.D., Zhang, W., Gruenheid, S., Gros, P., and Grinstein, S. (1998) Host resistance to intracellular infection: mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification. *J Exp Med* **188**: 351–364.

Harada, M., Sakisaka, S., Kawaguchi, T., Kimura, R., Taniguchi, E., Koga, H., *et al.* (2000) Copper does not alter the intracellular distribution of ATP7B, a copper-transporting ATPase. *Biochem Biophys Res Commun* **275**: 871–876.

Harris, E.D. (2000) Cellular copper transport and metabolism. *Annu Rev Nutr* **20**: 291–310.

Hill, N.J., Lyons, P.A., Armitage, N., Todd, J.A., Wicker, L.S., and Peterson, L.B. (2000) NOD Idd5 locus controls insulitis and diabetes and overlaps the orthologous CTLA4/IDDM12 and NRAMP1 loci in humans. *Diabetes* **49**: 1744–1777.

Hirsch, E.C., and Faucheux, B.A. (1998) Iron metabolism and Parkinson's disease. *Mov Disord* **13**: 39–45.

Hofmeister, A., Neibergs, H.L., Pokorny, R.M., and Galanduik, S. (1997) The natural resistance-associated macrophage protein gene is associated with Crohn's disease. *Surgery* **122**: 173–179.

Jabado, N., Jankowski, A., Dougaparsad, S., Picard, V., Grinstein, S., and Gros, P. (2000) Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane. *J Exp Med* **192**: 1237–1248.

Jellinger, K.A., Kienzl, E., Rumpelmaier, G., Paulus, W., Riederer, P., Stachelberger, H., *et al.* (1993) Iron and ferritin in substantia nigra in Parkinson's disease. *Adv Neurol* **60**: 267–272.

Kaye, P.M., and Blackwell. J.M. (1989) Lsh, antigen presentation and the development of CMI. *Res Immunol* **140**: 810–815.

Kehres, D.G., Zaharik, M.L., Finlay, B.B., and Maguire, M.E. (2000) The NRAMP proteins of *Salmonella typhimurium* and

Escherichia coli are selective manganese transporters involved in the response to reactive oxygen. *Mol Microbiol* **36**: 1085–1100.

Kramnik, I., Radzioch, D., and Skamene, E. (1994) T-helper 1-like subset selection in *Mycobacterium bovis* bacillus Calmette-Guérin-infected resistant and susceptible mice. *Immunology* **81**: 618–625.

Kuhn, D.E., Baker, B.D., Lafuse, W.P., and Zwilling, B.S. (1999) Differential iron transport into phagosomes isolated from the RAW264.7 macrophage cell lines transfected with Nramp1Gly169 or Nramp1Asp169. *J Leukoc Biol* **66**: 113–119.

Kuhn, D.E., Lafuse, W.P., and Zwilling, B.S. (2001) Iron transport into mycobacterium avium-containing phagosomes from an Nramp1 (Gly169)-transfected RAW264.7 macrophage cell line. *J Leukoc Biol* **69**: 43–49.

Lafuse, W.P., Alvarez, G.R., and Zwilling, B.S. (2000) Regulation of Nramp1 mRNA stability by oxidants and protein kinase C in RAW264.7 macrophages expressing Nramp1 (Gly169). *Biochem J* **351**: 687–696.

Lang, T., Prina, E., Sibthorpe, D., and Blackwell. J.M. (1997) Nramp1 transfection transfers Ity/Lsh/Bcg-related pleiotropic effects on macrophage activation: Influence on antigen processing and presentation. *Infect Immun* **65**: 380–386.

Leclercq, V., Lebastard, M., Belkaid, Y., Louis, J., and Milon, G. (1996) The outcome of the parasitic process initiated by *Leishmania infantum* in laboratory mice. a tissue-dependent pattern controlled by the Lsh and MHC loci. *J Immunol* **157**: 4537–4545.

Maliarik, M.J., Chen, K.M., Sheffer, R.G., Rybicki, B.A., Major, M.L., Popovich, J., Jr, and Iannuzzi, M.C. (2000) The natural resistance-associated macrophage protein gene in African Americans with sarcoidosis. *Am J Respir Cell Mol Biol* **22**: 672–675.

Marquet, S., Sanchez, F.O., Arias, M., Rodriguez, J., Paris, S.C., Skamene, E., *et al.* (1999) Variants of the human NRAMP1 gene and altered human immunodeficiency virus infection susceptibility. *J Infect Dis* **180**: 1521–1525.

Medina, E., and North, R.A. (1996a) The Bcg gene (Nramp1) does not determine resistance of mice to virulent *Mycobacterium tuberculosis*. *Ann NY Acad Sci* **797**: 257–259.

Medina, E., and North, R.J. (1996b) Evidence inconsistent with a role for the Bcg gene (Nramp1) in resistance of mice to infection with virulent *Mycobacterium tuberculosis*. *J Exp Med* **183**: 1045–1051.

Multhaup, G. (1997) Amyloid precursor protein, copper and Alzheimer's disease. *Biomed Pharmacother* **51**: 105–111.

North, R.J., LaCourse, R., Ryan, L., and Gros, P. (1999) Consequence of Nramp1 deletion to *Mycobacterium tuberculosis* infection in mice. *Infect Immun* **67**: 5811–5814.

Petris, M.J., and Mercer, J.F. (1999) The Menkes protein (ATP7A; MNK) cycles via the plasma membrane both in basal and elevated extracellular copper using a C-terminal di-leucine endocytic signal. *Hum Mol Genet* **8**: 2107–2115.

Pinner, E., Gruenheid, S., Raymond, M., and Gros, P. (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.

Portnoy, M.E., Liu, X.F., and Culotta, V.C. (2000) Saccharomyces cerevisiae expresses three functionally distinct homologues of the nramp family of metal transporters. *Mol Cell Biol* **20**: 7893–7902.

Puliti, M., Radzioch, D., Mazzolla, R., Barluzzi, R., Bistoni, F., and Blasi, E. (1995) Influence of the Bcg locus on macrophage response to the dimorphic fungus *Candida albicans*. *Infect Immun* **63**: 4170–4173.

Roach, T.I., Kiderlen, A.F., and Blackwell. J.M. (1991) Role of inorganic nitrogen oxides and tumor necrosis factor-alpha in killing *Leishmania donovani* amastigotes in gamma interferon-lipopolysaccharide-activated macrophages from Lsh^s and Lsh^r congenic mouse strains. *Infect Immun* **59**: 3935–3944.

Rodrigues, V., Cheah, V.P.Y., Ray, K., and Chia, W. (1995) malvolio, the *Drosophila* homologue of mouse Nramp1 (Bcg), is expressed in macrophages and in the nervous system and is required for normal taste behaviour. *EMBO J* **14**: 3007–3020.

Ryu, S., Park, Y.K., Bai, G.H., Kim, S.J., Park, S.N., and Kang, S. (2000) 3′UTR. polymorphisms in the NRAMP1 gene are associated with susceptibility to tuberculosis in Koreans. *Int J Tuberc Lung Dis* **4**: 577–580.

Sanjeevi, C.B., Miller, E.N., Dabadghao, P., Rumba, I., Shtauvere, A., Denisova, A., *et al.* (2000) Polymorphism at NRAMP1 and D2S1471 loci associated with juvenile rheumatoid arthritis. *Arthritis Rheum* **43**: 1397–1404.

Searle, S., and Blackwell. J.M. (1999) Evidence for a functional repeat polymorphism in the promoter of the human NRAMP1 gene that correlates with autoimmune versus infectious disease susceptibility. *J Med Genet* **36**: 295–299.

Searle, S., Bright, N.A., Roach, T.I.A., Atkinson, P.G.P., Barton, C.H., Meloen, R.H., and Blackwell. J.M. (1998) Localisation of Nramp1 in macrophages: modulation with activation and infection. *J Cell Sci* **111**: 2855–2866.

Shaw, M.-A., Clayton, D., Atkinson, S.E., Williams, H., Miller, N., Sibthorpe, D., and Blackwell. J.M. (1996) Linkage of rheumatoid arthritis to the candidate gene NRAMP1 on 2q35. *J Med Genet* **33**: 672–677.

Shaw, M.-A., Collins, A., Peacock, C.S., Miller, E.N., Black, G.F., Sibthorpe, D., *et al.* (1997) Evidence that genetic susceptibility to *Mycobacterium tuberculosis* in a Brazilian population is under oligogenic control: linkage study of the candidate genes NRAMP1 and TNFA. *Tuberc Lung Dis* **78**: 35–45.

Shiloh, M.U., MacMicking, J.D., Nicholson, S., Brause, J.E., Potter, S., Marino, M., *et al.* (1999) Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* **10**: 29–38.

Shirakawa, T., Enomoto, T., and Shimazu, S.-I., and Hopkin, J.M. (1997) The inverse association between tuberculin responses and atopic disorder. *Science* **275**: 77–79.

Singal, D.P., Li, J., Zhu, Y., and Zhang, G. (2000) NRAMP. 1 gene polymorphisms in patients with rheumatoid arthritis. *Tissue Antigens* **55**: 44–47.

Skamene, E., Gros, P., Forget, A., Patel, P.J., and Nesbitt, M.N. (1984) Regulation of resistance to leprosy by chromosome 1 locus in the mouse. *Immunogenetics* **19**: 117–124.

Soo, S.-S., Villarreal Ramos, B., Khan, C.M., Hormaeche, C.E., and Blackwell, J.M. (1998) Genetic control of immune response to recombinant antigens carried by an attenuated *Salmonella typhimurium* vaccine strain: Nramp1 influences T-helper subset responses and protection against leishmanial challenge. *Infect Immun* **66**: 1910–1917.

Stern, J.J., Oca, M.J., Rubin, B.Y., Anderson, S.L., and Murray, H.W. (1988) Role of L3T4+ and LyT-2+ cells in experimental visceral leishmaniasis. *J Immunol* **140**: 3971–3977.

Strausak, D., La Fontaine, S., Hill, J., Firth, S.D., Lockhart, P.J., and Mercer, J.F. (1999) The role of GMXCXXC metal binding sites in the copper-induced redistribution of the Menkes protein. *J Biol Chem* **274**: 11170–11177.

Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., *et al.* (1994) Lack of acidification

in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* **263**: 678–681.

Sturgill-Koszycki, S., Schaible, U.E., and Russell, D.G. (1996) *Mycobacterium*-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. *EMBO J* **15**: 6960–6968.

Supeck, F., Supekova, L., Nelson, H., and Nelson, N. (1996) A. yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. *Proc Natl Acad Sci USA* **93**: 5105–5110.

Tabuchi, M., Yoshida, T., Takegawa, K., and Kishi, F. (1999) Functional analysis of the human NRAMP family expressed in fission yeast. *Biochem J* **344**: 211–219.

Vanderwerf, S., Cooper, M.J., Stetsenko, I.V., and Lutsenko, S. (2001) Copper specifically regulates intracellular phosphorylation of the Wilson's disease protein, a human copper-transporting ATPase. *J Biol Chem* **26**: July 26 (epub).

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D.W., Lucia, S.M., Dinauer, M.C., *et al.* (2000) *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* **287**: 1655–1658.

Vidal, S.M., Malo, D., Vogan, K., Skamene, E., and Gros, P. (1993) Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell* **73**: 469–485.

Vidal, S., Tremblay, M.L., Govoni, G., Gauthier, S., Sebastiani, G., Malo, D., *et al.* (1995) The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *J Exp Med* **182**: 655–666.

Voskoboinik, I., Strausak, D., Greenough, M., Brooks, H., Petris, M., Smith, S., *et al.* (1999) Functional analysis of the N-terminal CXXC metal-binding motifs in the human Menkes copper-transporting P-type ATPase expressed in cultured mammalian cells. *J Biol Chem* **274**: 22008–22012.

Wei, X.Q., Charles, I.G., Smith, A., Ure, J., Feng, G.J., Huang, F.P., *et al.* (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* **375**: 408–411.

Yang, Y.S., Kim, S.J., Kim, J.W., and Koh, E.M. (2000) NRAMP. 1 gene polymorphisms in patients with rheumatoid arthritis in Koreans. *J Korean Med Sci* **15**: 83–87.

Yuan, X.M., Brunk, U.T., and Olsson, A.G. (1995) Effects of iron- and hemoglobin-loaded human monocyte-derived macrophages on oxidation and uptake of LDL. *Arterioscl Throm Vas* **15**: 1345–1351.

Yuan, X.M., Anders, W.L., Olsson, A.G., and Brunk, U.T. (1996) Iron in human atheroma and LDL oxidation by macrophages following erythrophagocytosis. *Atherosclerosis* **124**: 61–73.

Zwilling, B.S., Kuhn, D.E., Wikoff, L., Brown, D., and LaFuse, W. (1999) The role of iron in Nramp1 mediated inhibition of mycobacterial growth. *Infect Immun* **67**: 1386–1392.