

Solute carrier 11a1 (*Slc11a1*; formerly *Nramp1*) regulates metabolism and release of iron acquired by phagocytic, but not transferrin-receptor-mediated, iron uptake

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Solute carrier 11a1 (*Slc11a1*; formerly *Nramp1*; where *Nramp* stands for natural-resistance-associated macrophage protein) is a proton/bivalent cation antiporter that localizes to late endosomes/lysosomes and controls resistance to pathogens. In the present study the role of *Slc11a1* in iron turnover is examined in macrophages transfected with *Slc11a1*^{Gly169} (wild-type) or *Slc11a1*^{Asp169} (mutant = functional null) alleles. Following direct acquisition of transferrin (Tf)-bound iron via the Tf receptor, iron uptake and release was equivalent in wild-type and mutant macrophages and was not influenced by interferon- γ /lipopolysaccharide activation. Following phagocytosis of [⁵⁹Fe]Tf-anti-Tf immune complexes, iron uptake was equivalent and up-regulated similarly with activation, but intracellular distribution was markedly different. In wild-type macrophages most iron was in the soluble (60%) rather than insoluble (12%) fraction, with 28% ferritin (Ft)-bound. With activation, the soluble component increased to 82% at the expense of Ft-bound iron (< 5%). In

mutant macrophages, 40–50% of iron was in insoluble form, 50–60% was soluble and < 5% was Ft-bound. Western-blot analysis confirmed failure of mutant macrophages to degrade complexes 24 h after phagocytic uptake. Confocal microscopy showed that complexes were within lysosome-associated membrane protein 1-positive vesicles in wild-type and mutant macrophages at 30 min and 24 h, implying failure in the degradative process in mature phagosomes in mutant macrophages. NO-mediated iron release was 2.4-fold higher in activated wild-type macrophages compared with mutant macrophages. Overall, our data suggest that iron acquired by phagocytosis and degradation is retained within the phagosomal compartment in wild-type macrophages, and that NO triggers iron release by direct secretion of phagosomal contents rather than via the cytoplasm.

Key words: macrophage, nitric oxide, phagocytosis.

INTRODUCTION

Macrophages play a critical role in iron metabolism. They are responsible for the processing of haemoglobin-iron from senescent erythrocytes and subsequent iron supply to the bone marrow for erythropoiesis [1]. In addition, iron is essential in macrophage-mediated cytotoxicity by contributing to the production of highly toxic hydroxyl radicals via the Fenton reaction [2], and by controlling the production of NO after activation by immunological stimuli [3]. Two new transporters of ferrous iron have been identified. Solute carrier 11a2 (*Slc11a2*; formerly *Nramp2*; where *Nramp* corresponds to natural-resistance-associated macrophage protein) localizes to early recycling endosomes and is ubiquitous in expression, including macrophages [4]. *Slc11a1* (formerly *Nramp1*) localizes to late endosomes/lysosomes [5,6] and is more restricted in cellular distribution, principally to cells of the myeloid lineage. Both are polytopic integral membrane proteins with 10–12 predicted membrane-spanning domains. However, whereas *Slc11a2* is a symporter of protons and bivalent cations, *Slc11a1* is an antiporter that fluxes bivalent cations in either direction depending on the pH on either side of the membrane [7]. Hence, under physiological conditions, *Slc11a2* is expected to

transport bivalent cations from early endosomes to the cytoplasm, whereas *Slc11a1* will transport bivalent cations from the cytoplasm into late endosomes, lysosomes and phagosomes. In terms of iron uptake and metabolism within macrophages, this places *Slc11a2* in a location likely to influence transferrin (Tf)-receptor-mediated entry of iron into cells, whereas *Slc11a1* would be expected to influence degradation and iron recycling from effete erythrocytes entering macrophages by phagocytosis. To test this hypothesis, we compare the fate of intracellular iron acquired either directly through the Tf receptor as Tf-bound iron or following phagocytosis of [⁵⁹Fe]Tf-anti-Tf immune complexes in RAW264.7 macrophage cell lines stably transfected with either *Slc11a1*^{Gly169} (wild-type) or *Slc11a1*^{Asp169} (mutant = functional null) alleles.

EXPERIMENTAL

Reagents

RPMI 1640 culture medium, antibiotics, PBS and fetal calf serum (FCS) were purchased from Gibco (Paisley, Renfrewshire, Scotland, U.K.). Human apoTf was obtained from Sigma (Poole, Dorset, U.K.) and, when required, saturated with ⁵⁹Fe using Fe-

Abbreviations used: EEA1, early endosome antigen 1; FCS, fetal calf serum; Ft, ferritin; IFN γ , interferon- γ ; IRP, iron-regulatory protein; LAMP1, lysosome-associated membrane protein 1; LPS, lipopolysaccharide; L-NMMA, N^G-monomethyl-L-arginine; *Nramp*, natural-resistance-associated macrophage protein; NTA, nitrilotriacetate; *Slc11a1/2*, solute carrier 11a1/2; Tf, transferrin.

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nitrilotriacetate (NTA; Fe/NTA molar ratio of 1:4) [8] prepared from Na-NTA and $^{59}\text{FeCl}_3$ (specific radioactivity of 3–10 mCi/mg; NEN Life Science Products, Boston, MA, U.S.A.). Human serum albumin (Tf-free), *N*^G-monomethyl-L-arginine (L-NMMA), lipopolysaccharide (LPS), leupeptin, PMSF, benzamidine and pepstatin A were all obtained from Sigma. Nitrocellulose membranes and ECL[®] reagents were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). FITC-conjugated annexin V was obtained from Pharmingen (San Diego, CA, U.S.A.), and rabbit anti-(human Tf) was purchased from Dako (Glostrup, Denmark). Rabbit anti-[mouse ferritin (Ft)] was produced in our laboratory [8]. Mouse recombinant interferon- γ (IFN γ) was kindly provided by Dr G. Adolf (Bender, Vienna, Austria).

Preparation of immune complexes

For radiolabel studies, ^{59}Fe -NTA was added in a quantity sufficient to saturate the iron binding capacity of human apoTf and then optimal proportions of Tf and anti-Tf (normally 1 mg of Tf to 2.5 ml of anti-Tf) were mixed and incubated at 50 °C for 1 h [9]. The insoluble immune complexes (^{59}Fe]Tf-antiTf) were centrifuged at 500 g for 5 min, washed twice in PBS and resuspended in PBS. For confocal microscopy, cold Fe-NTA was used to prepare immune complexes, which were labelled with FITC by conjugating the Tf using the Sigma Fluorotag kit.

Macrophage cultures and treatments

The RAW264.7 mouse macrophage cell lines stably transfected with wild-type *Slc11a1*^{G1y169} (7.5R) or mutant *Slc11a1*^{Asp169} (10S) alleles [10] were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 100 units/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin.

Macrophages were stimulated overnight with 50 units/ml IFN γ and 10 ng/ml LPS, pulsed for 2 h with 10 $\mu\text{g/ml}$ ^{59}Fe]Tf-antiTf immune complexes, washed twice with PBS, incubated for 48 h in the presence of the same additives and subjected to further procedures as indicated below. In some experiments FCS was replaced by BSA (0.2%) and the cells pulsed for 2 h with either ^{59}Fe -citrate (1 μM Fe) or ^{59}Fe]Tf (10 μM). Cells were fed with fresh medium containing the appropriate additives every 24 h, and viability was checked by Trypan Blue exclusion.

Immunostaining and confocal microscopy

To determine the intracellular fate of immune complexes, macrophages were loaded with 10 $\mu\text{g/ml}$ FITC-labelled Tf-antiTf, fixed at intervals and immunostained with antibodies raised against early endosome {goat polyclonal anti-[early endosome antigen 1 (EEA1)]; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.} or late endosomes/lysosome {rat monoclonal anti-[lysosome-associated membrane protein 1 (LAMP1)] ID4B; prepared in-house} markers. Briefly, 5×10^4 wild-type or mutant macrophages in 400 μl were plated into the wells of chamber slides (Labtech; distributed through Gibco) and allowed to settle overnight prior to uptake of immune complexes. Cells were washed in serum-free medium and then incubated in serum-free medium with immune complexes for 1 h at 37 °C. Cells were washed in PBS and either harvested and fixed then, or incubated in RPMI/10% (v/v) FCS for a further 24 h. Cells were washed in PBS and fixed in ice-cold methanol for 20 min and air-dried. Slides were stored at -20 °C. For immunofluorescent staining, cells were rehydrated in PBS and blocked for 1 h at room temperature (21–24 °C) in either PBS containing 5% (w/v) dried milk powder and 0.1% Triton X-100 (for LAMP1) or PBS

containing 3% (v/v) horse serum and 0.1% Triton X-100 (for EEA1). Cells were incubated in a 1:100 dilution of primary antibody in an appropriate blocking solution overnight at 4 °C, washed three times for 10 min at room temperature in PBS containing 0.1% Triton X-100, and incubated for 2 h at room temperature in a 1:200 dilution of secondary antibody (goat anti-rat Alexa Fluor 594, red, for LAMP1; and donkey anti-goat Alexa Fluor 594, red, for EEA1; Molecular Probes, Europe BV, Leiden, The Netherlands) in appropriate blocking solution. Slides were washed two times for 5 min in PBS containing 0.1% Triton X-100, washed three times for 5 min in PBS, and mounted in Vectorshield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.). Preparations were examined using confocal microscopy as previously described [6].

Iron uptake and release assays

Release of iron into the medium was monitored by determining ^{59}Fe activity in the culture medium at 24 and 48 h intervals using an LKB Compugamma counter. At the end of the experiments, macrophages were lysed in 2% (w/v) SDS, and the remaining cell-associated radioactivity was determined.

Intracellular distribution of iron

Cells were lysed in 200 μl of cytoplasmic lysis buffer [25 mM Tris/HCl (pH 7.4), 1% (v/v) Triton X-100, 40 mM KCl, 50 $\mu\text{g/ml}$ leupeptin, 200 $\mu\text{g/ml}$ PMSF, 1 mM benzamidine and 50 $\mu\text{g/ml}$ pepstatin A] and the intracellular iron distribution was determined as described previously [11]. Briefly, this consisted of centrifugation at 10000 g for 5 min and immunoprecipitation of Ft with a rabbit anti-Ft antibody. This allows separation of intracellular iron into three compartments containing predominantly insoluble material (consisting mainly of undegraded immune complexes and iron bound to intracellular organelles), Ft-bound iron and non-Ft soluble iron.

Estimation of Ft

Macrophages stimulated as described above and pulsed if required with unlabelled immune complexes were lysed with 50–100 μl of boiling lysis solution [1% (w/v) SDS/10 mM Tris/HCl (pH 7.4)]. Aliquots containing 5–10 μg of protein were analysed by Western blotting using a 15% (w/v) acrylamide gel and overnight transfer at 150 mA to nitrocellulose membranes. The blots were developed using rabbit anti-(mouse Ft) antibody and ECL[®] (Amersham Biosciences) according to the manufacturer's protocol.

Estimation of apoptotic cells

The percentage of cells undergoing apoptosis was determined by flow cytometry using a FITC-annexin-5 kit (Genzyme, Cambridge, MA, U.S.A.) according to the manufacturer's instructions.

Measurement of nitrite

Nitrite in the culture medium was determined using the Griess reagent.

Protein determination

The protein concentration of cell lysates was estimated by the bicinchoninic acid ('BCA') protein assay reagent (Pierce, Chester, Cheshire, U.K.) using BSA as a standard.

Statistical analysis

Data were analysed by one-way ANOVA and an unpaired Student's *t* test to determine the difference between each group. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Uptake, release and intracellular distribution of iron acquired from [⁵⁹Fe]Tf-anti-Tf immune complexes or [⁵⁹Fe]Tf in *Slc11a1*-transfected macrophages

To determine the effect of *Slc11a1* expression on cellular iron handling, RAW264.7 macrophage cell lines transfected with wild-type *Slc11a1*^{Gly169} or mutant *Slc11a1*^{Asp169} alleles were stimulated with IFN γ and LPS, and then pulsed with [⁵⁹Fe]Tf-anti-Tf immune complexes or [⁵⁹Fe]Tf. Stimulation of macrophages with IFN γ and LPS enhanced uptake of immune complexes in both mutant and wild-type *Slc11a1* macrophages (Figure 1a), the effect being independent of NO. Interestingly, however, IFN γ - and LPS-activated wild-type *Slc11a1* macrophages showed a 2.4-fold enhancement ($P < 0.001$) in the amount of iron released compared with mutant *Slc11a1* macrophages (Figure 1b). The trigger for this release was inhibited by L-NMMA ($P < 0.01$ for mutant cells; and $P < 0.001$ for wild-type cells) and was therefore NO-dependent (Figure 1b). In contrast, no differences in iron uptake (results not shown) or release (Figure 1c) were observed between wild-type and mutant macrophages when iron was delivered to the macrophages as [⁵⁹Fe]Tf. This is consistent with the observation that *Slc11a1* is expressed in late endosomes/lysosomes and phagolysosomes, but not in the

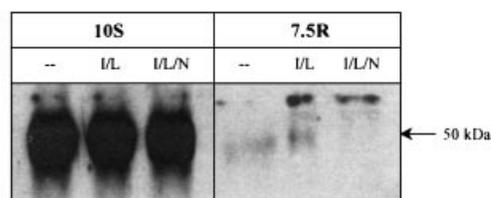


Figure 2 Effect of *Slc11a1* on degradation of FeTf-anti-Tf immune complexes by transfected RAW264.7 macrophages

RAW264.7 macrophages transfected with either wild-type *Slc11a1* (7.5R) or the non-functional Gly¹⁶⁹ \rightarrow Asp mutant (10S) were stimulated, pulsed with unlabelled FeTf-anti-Tf immune complexes, and after a further 24 h the cells were lysed and the presence of rabbit IgG from the anti-Tf antibody was determined by Western-blot analysis. The 50 kDa bands represent the Ig heavy chains of undegraded immune complexes.

early recycling endosome compartment where iron is acquired from Tf via endocytosis of the Tf-Tf receptor. To eliminate the possibility that the increased iron release from activated wild-type-transfected macrophages pulsed with [⁵⁹Fe]Tf-anti-Tf might be due to leakage from moribund (dying) cells, the degree of apoptosis was measured 24 h after pulsing. This was found to be similar in both wild-type (0.5% and 0.6% for [⁵⁹Fe]Tf-anti-Tf alone; 4.9% and 4.6% for [⁵⁹Fe]Tf-anti-Tf with IFN γ /LPS; $n = 2$) and mutant (0.9% and 1.2% for [⁵⁹Fe]Tf-anti-Tf alone; 5.3% and 4.2% for [⁵⁹Fe]Tf-anti-Tf with IFN γ /LPS; $n = 2$) cell lines, indicating that increased leakage from a larger number

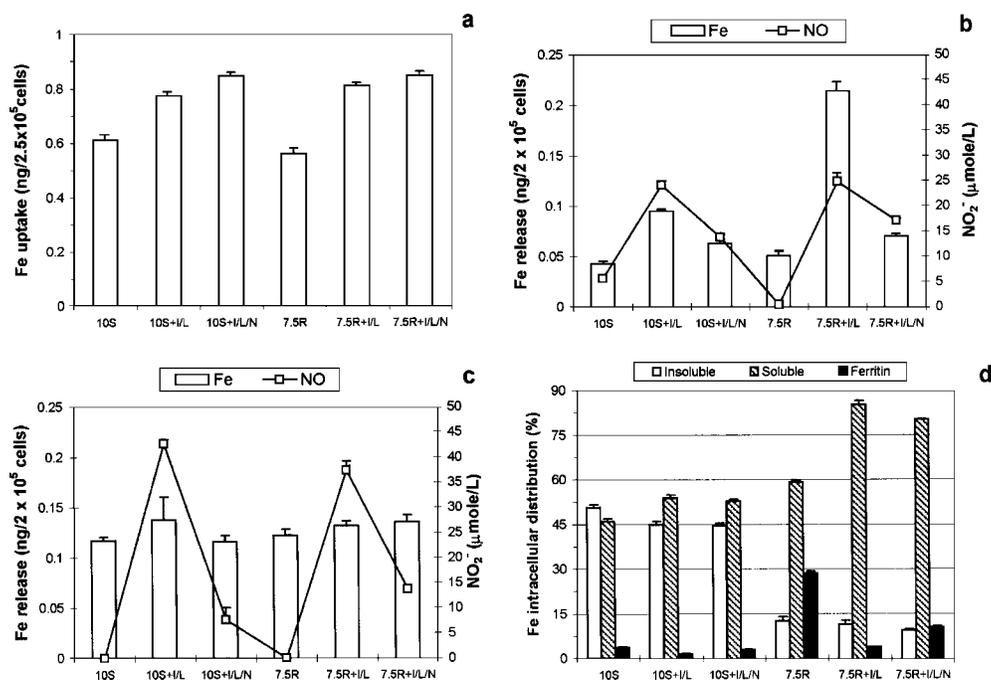


Figure 1 Effect of *Slc11a1* on uptake, release and intracellular distribution of iron taken up as [⁵⁹Fe]Tf-anti-Tf immune complexes or from [⁵⁹Fe]Tf by transfected RAW264.7 macrophages

RAW264.7 macrophages transfected with either wild-type *Slc11a1* (7.5R) or the non-functional Gly¹⁶⁹ \rightarrow Asp mutant (10S) were stimulated with IFN γ /LPS (I/L), with or without L-NMMA (N), or left unstimulated. Cells were pulsed with [⁵⁹Fe]Tf-anti-Tf immune complexes (a, b and d) or [⁵⁹Fe]Tf (c) for 2 h, and iron uptake (a), iron release (b and c) and intracellular distribution (d) were determined at 24 h post-pulse. NO production was measured 24 h (b and c) after pulsing.

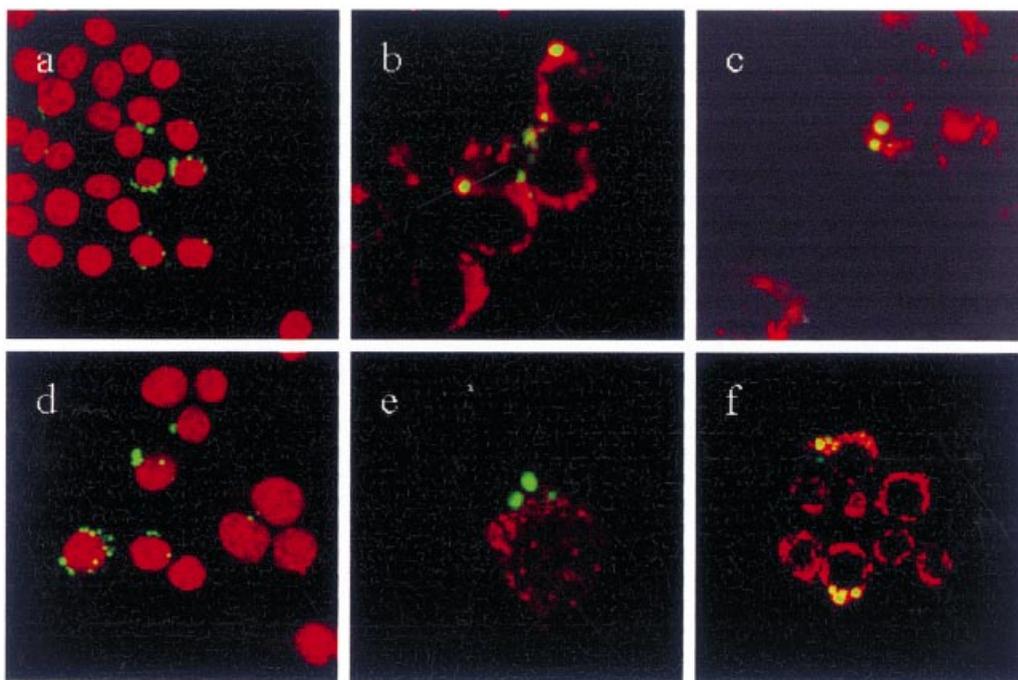


Figure 3 Co-localization of FITC-labelled FeTf-anti-Tf immune complexes with early endosomal (EEA1) versus late endosome/lysosome (LAMP1) markers in transfected RAW264.7 macrophages

RAW264.7 macrophages transfected with either the non-functional Gly¹⁶⁹ → Asp mutant (10S) (**a, b and c**) or wild-type (7.5R) (**d, e and f**) Slc11a1 were pulsed with immune complexes for 30 min, and were examined by confocal microscopy at 30 min (**a, b and d**) or 24 h (**c, e and f**). Green FITC-labelled complexes in cells with nuclei stained with propidium iodide. Immune complexes (green) were located within LAMP1-positive (red) vesicles in mutant (**b and c**) and wild-type (**f**) macrophages at 30 min (**b**) and 24 h (**c and f**). Vesicles containing immune complexes (green) were almost exclusively negative for EEA1 (red) in both wild-type (**e**) and mutant (results not shown) macrophages at both 30 min (results not shown) and 24 h (**e**).

of moribund cells in the wild-type-transfected cells is not the reason for the difference in iron release.

To further investigate the role of Slc11a1 in macrophage iron metabolism, we next investigated the distribution of incorporated iron into the two macrophage cell lines. At 24 h after the [⁵⁹Fe]Tf-anti-Tf pulse, mutant-transfected macrophages contained approx. 50% insoluble iron, whereas wild-type-transfected macrophages contained only approx. 10% insoluble iron irrespective of the state of activation or NO production (Figure 1d). Western-blot analysis revealed that the mutant-, but not the wild-type-, transfected cells still contained a substantial amount of intact rabbit IgG (Figure 2), indicative of undegraded immune complexes. Using confocal microscopy we were able to detect fluorescent immune complexes in vesicles of both Gly¹⁶⁹ → Asp mutant (Figure 3a) and wild-type (Figure 3d) macrophages. Immunostaining showed that immune complexes co-localized with LAMP1 at 30 min (Figure 3b) and 24 h (Figures 3c and 3f) in both Slc11a1 wild-type and mutant cells, with no immune complexes held up in the EEA1-positive (Figure 3e) early endosomal compartment. Mutant-transfected cells therefore appear to be much less efficient at degrading immune complexes in the late endosomal/lysosomal compartment, consistent with previous studies [12] demonstrating reduced delivery of vacuolar-ATPase and acidification of phagosomes in mutant compared with wild-type macrophages. In these cells, iron in the insoluble fraction probably corresponded to ⁵⁹Fe still bound to undegraded insoluble immune complexes, rather than to mitochondria. In contrast, the generally higher soluble non-Ft fraction in the wild-type-transfected macrophages probably consists of iron that has

been released from the immune complexes but is still within the phagosome.

The proportion of Ft-bound iron was higher in wild-type macrophages ($P < 0.0001$), with IFN γ and LPS activation causing a marked decrease ($P < 0.0001$) that was partially reversed by L-NMMA ($P < 0.0001$). A similar effect was seen in the mutant-transfected macrophages, although the overall proportions of Ft-bound iron were much lower. These results indicate that Slc11a1 promotes iron release from the immune complexes and its subsequent binding by Ft, whereas NO switches the balance towards iron release rather than incorporation into Ft.

Ft expression by Slc11a1-transfected macrophages

To investigate possible mechanisms responsible for the differences in intracellular distribution of iron in mutant- and wild-type-transfected macrophages, we examined Ft expression by the two cell lines. In the first place, it is evident that the mutant-transfected cells contained more Ft than the wild-type-transfected cells, since an exposure of the film that allowed demonstration of regulation of Ft in the mutant-transfected cells gave no bands for the wild-type-transfected cells (Figure 4a). It can be seen that IFN γ and LPS down-regulated Ft expression in the mutant-transfected cells, the effect being reversed by L-NMMA (Figure 4a). When the film was overexposed for the mutant-transfected cells in order to allow visualization of Ft in the wild-type-transfected cells, it became evident that although the Ft levels were much lower they were regulated by IFN γ /LPS and L-

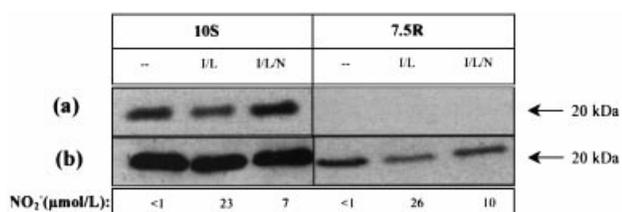


Figure 4 Ft in transfected RAW264.7 macrophages

RAW264.7 macrophages transfected with either wild-type *Slc11a1* (7.5R) or the non-functional Gly¹⁶⁹ → Asp mutant (10S) were treated as in Figure 1, and Ft expression was determined by Western-blot analysis. NO was measured in the culture supernatant at the time of lysis. In (a) the autoradiograph was exposed for 1 min; (b) shows the same autoradiograph exposed for 5 min. The 20 kDa bands represent the heavy (21 kDa) and light (20.8 kDa) chains of Ft that co-migrate.

NMMA as in the mutant cells (Figure 4b). These results indicate that, despite the large overall difference in Ft levels between the two cell lines, in each cell line Ft appears to be down-regulated by NO. This is consistent with results we have obtained comparing bone marrow macrophages from *Nos2A* wild-type and *Nos2A*-deficient mice (where *Nos2A* is the gene for nitric oxide synthase 2A; V. Mulero, X.-q. Wei, F. Y. Liew and J. H. Brock, unpublished work). However, this effect is overshadowed by the large differences in Ft that are evidently associated with *Slc11a1* function.

DISCUSSION

It is well known that mononuclear phagocytes of the liver and spleen acquire iron as a result of erythrophagocytosis during the normal process of removal of effete erythrocytes. However, the mechanisms involved in the liberation of iron by these cells in order for it to be returned to the circulation remain unclear [1]. In the present study we report on studies using uptake of iron via [⁵⁹Fe]Tf-anti-Tf immune complexes as a model of iron recycling via erythrophagocytosis. We demonstrate that when iron is delivered to late endosomes/lysosomes via this phagocytic pathway, but not via the Tf receptor in the early recycling endosomes, macrophages stably transfected with the wild-type allele of *Slc11a1* recycle 2.4-fold more iron to the medium than mutant macrophages. Release of iron is inhibitable by L-NMMA, indicating that NO provides a crucial signal for this iron release. These studies suggest that *Slc11a1* plays an important role in recycling of iron acquired by macrophages by phagocytosis, implying a role in degradation and recycling of iron from effete erythrocytes. This is of interest in the light of the role of *SLC11A1* in infectious and autoimmune disease susceptibility (reviewed in [13]), where retention of iron by, or excess deposition of iron around, macrophages is associated with inflammation [14] and can lead to the anaemia of chronic disease [15,16].

It has generally been assumed that iron released by macrophages must first enter the cytoplasm from the phagosome for subsequent export across the cytoplasmic membrane, perhaps by a protein analogous to the recently described ferroportin [17], also known to be expressed in macrophages [18]. In terms of *Slc11a1* activity, this pathway would be consistent with the work of Jabadó et al. [19], who used a bivalent-cation-sensitive fluorescent probe covalently attached to zymosan particles to show that phagosomes from *Slc11a1*^{Gly169} macrophages extrude Mn²⁺ faster than *Slc11a1*-deficient macrophages. This was demonstrated by measuring the rate of quenching of particle-

bound fluorescence following addition of a non-physiological level (500 μM) of exogenous Mn²⁺. The efflux of Mn²⁺ from phagosomes under these conditions could be explained by our earlier observation [7] that *Slc11a1* can flux bivalent cations in either direction depending upon the pH, and presumably bivalent-cation concentration, on either side of the membrane. An interesting facet of our results in the present study is that, under physiological conditions of low intravacuolar pH, the weight of evidence [7,20,21] supports the hypothesis that *Slc11a1* transports bivalent cations into, rather than out of, late endosomes/lysosomes. Hence, a route out of the cell via the cytoplasm for iron acquired by phagocytosis by *Slc11a1* wild-type macrophages seems unlikely. This raises the possibility that iron is released from macrophages directly via a lysosomal secretory pathway. Given the potentially damaging influence of high cytoplasmic iron on mRNA stability [22], this may prove the safest route for recycling of iron from effete erythrocytes. Our results are consistent with earlier studies [23] showing that 40% of iron acquired from erythrocytes by isolated Kupffer cells, one of the primary sites of *Slc11a1* regulated antimicrobial activity [24], is released within 24 h. However, one issue that remains unclear in this process is the extent to which the release of iron from haem following erythrocyte digestion in macrophages is dependent upon haem oxygenase, and whether this requires cycling via the cytoplasm to access haem oxygenase thought to localize on the cytoplasmic face of the endoplasmic reticulum [25]. Erythrophagocytosis, but not direct iron treatment, has been shown to induce haem oxygenase in human blood monocytes [26]. The localization of haem iron and haem oxygenase in *Slc11a1* wild-type and mutant macrophages following erythrophagocytosis would certainly be an area for further fruitful experimentation. Interestingly, we observe that, with macrophage activation, NO triggers the release of iron to the extracellular milieu following phagocytic uptake of iron in our model system. Secretion of lysosomal contents is known to occur in haemopoietic cells [27], and although there appears to be no data relating specifically to macrophages, NO is known to enhance secretory and/or excretory mechanisms in other cells [28,29], which could account for its ability to enhance iron release in the present study. Likewise the failure of mutant *Slc11a1*-transfected cells to release iron could be explained by defective delivery of the vacuolar-ATPase and phagosomal acidification [12], leading to an inability both to degrade immune complexes and to participate in the secretory process. Direct extracellular secretion of iron from phagosomal contents would also imply that most if not all iron entering a cell by phagocytosis never enters the cytoplasm and therefore does not contribute to the cytoplasmic iron pool responsible for regulating iron-regulatory protein (IRP) activity. Loading the cells with iron via Tf-anti-Tf immune complexes had little effect on IRP activity (results not shown) or Ft expression (Figure 4), compatible with this proposal. Although some radioactive iron was found in Ft, suggesting that some ingested iron can reach the cytoplasm, the amounts are relatively small compared with those seen in J774 cells loaded with Tf [11] and could be explained by the presence of Ft in phagosomes, as it is known that autophagy of Ft can occur [30]. Another factor that may influence iron release is that the mutant cells contained much more Ft than the wild-type cells, even though in both cases Ft levels appeared to be regulated in the same way in response to activation. It has been previously reported that the mutant cells contain more iron than the wild-type cells [31], and that wild-type cells have increased IRP2 activity [32]. This would explain the high levels of Ft in the mutant cells.

Overall our data provide concrete evidence that *Slc11a1* participates in release of iron taken up by macrophages through

phagocytosis. While Slc11a1 probably plays a central role in the maintenance of iron homeostasis in the steady state, its up-regulated expression with macrophage activation [6] and known associations with infectious and autoimmune diseases (reviewed in [13]) make it a primary candidate in regulating the anaemias of chronic disease.

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