Solute carrier protein family 11 member 1 (Slc11a1) activation efficiently inhibits *Leishmania donovani* survival in host macrophages

Nisha Singh^{1,2} · Mallikarjuna Rao Gedda¹ · Neeraj Tiwari¹ ·
Suya P. Singh¹ · Surabhi Bajpai^{1,3} · Rakesh K. Singh¹

Received: 31 August 2016 / Accepted: 8 November 2016 / Published online: 12 November 2016
© Indian Society for Parasitology 2016

Abstract Visceral leishmaniasis (kala-azar), a life threatening disease caused by *L. donovani*, is a latent threat to more than 147 million people living in disease endemic South East Asia region of the Indian subcontinent. The therapeutic option to control leishmanial infections are very limited, and at present comprise only two drugs, an antifungal amphotericin B and an antitumor miltefosine, which are also highly vulnerable for parasitic resistance. Therefore, identification and development of alternate control measures is an exigent requirement to control leishmanial infections. In this study, we report that functionally induced expression of solute carrier protein family 11 member 1 (Slc11a1), a transmembrane divalent cationic transporter recruited on the surface of phagolysosomes after phagocytosis of parasites, effectively inhibits *Leishmania donovani* growth in host macrophages. Further, the increased Slc11a1 functionality also resulted in increased production of NOx, TNF- α and IL-12 by activated macrophages. The findings of this study signify the importance of interplay between Slc11a1 expression and macrophages activation that can be effectively used to control of *Leishmania* growth and survival.

Keywords Slc11a1 · Macrophage · *Leishmania* · Survival

Introduction

Solute carrier protein family 11 member 1 (Slc11a1), also known as natural resistance associated macrophage protein1 (Nramp1), recruited to phagosomal membrane soon after phagocytosis of intracellular parasites by host macrophages (Gruenheid et al. 1995, Cellier et al. 2007). It is a transmembrane transporter efflux pump present on LAMP+ lysosome and endosomes of macrophages that pump out divalent cations from phagolysosomal milieu (Forbes and Gros 2001, Fritsche et al. 2007). The purpose of this recruitment is to create Fe²⁺, Zn²⁺, Mn²⁺ deprived conditions inside phagolysosomes of macrophages to ensure their least availability for parasites that eventually inhibit their growth (Jabado et al. 2000, Appelberg 2006). These cations, especially iron, are essentially required by parasites enzymatic machineries involved in their replication, growth, virulence as well as protection from oxidative damage produced by host oxidative stress mechanisms (Huynh and Andrews 2008).

Slc11a1 primarily acts as a proton dependent transporter that directly inhibits parasitic growth by removal divalent cations from parasitic milieu and indirectly exerts pleiotropic effects on both, innate and acquired immune functions (Stober et al. 2007). Activation of Slc11a1 has been shown to confer resistance against intracellular pathogens through stimulation of several antimicrobial effectors pathways including NADPH oxidase activity and NO formation, which are mainly controlled by intracellular iron availability (Forbes and Gros 2001). Various studies supports that the ion transport through Slc11a1 modulates the

✉ Rakesh K. Singh
rakesh_bc@bhu.ac.in; rakeshbhu@yahoo.com

¹ Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi 221005, India

² Present Address: Department of Biochemistry, HNB University, Srinagar, Uttarakhand 246174, India

³ Present Address: Department of Bioscience and Biotechnology, Banasthali University, Banasthali, Tonk, Rajasthan 304022, India

production of chemokines such as macrophage inflammatory protein 1 α (Mip-1 α), formation of reactive oxygen and nitrogen species (ROS and RNS), as well as antigen processing and presentation by host phagocytic cells (Blackwell et al. 2003, Fritsche et al. 2003, Stober et al. 2007, Valdez et al. 2008). Further, Slc11a1 functionality also promotes secretion of pro-inflammatory cytokines such as IFN- γ , TNF- α by phagocytic cells during pathogenic infections (Weiss et al. 1994, Fritsche et al. 2003). Increased Slc11a1 expression has also shown to direct Th1/Th2 dichotomy towards Th1 polarization to produce inflammatory cytokines such as IFN γ , IL-12 etc. along with decreased IL-10, a Th2 cytokine, production (Caron et al. 2006, Dai et al. 2009).

In *L. donovani* infection, macrophages are the major effector innate immune cells to control parasite growth in the host by generation of reactive oxidants and inflammatory cytokines. However, soon after phagocytosis *Leishmania* efficiently suppresses macrophages effectors functions and survives in its hostile environment. The parasites defense machinery effectively neutralizes ROS and RNI metabolites, resist to low pH conditions, and acquires nutrients from host macrophages for their growth (Liese et al. 2008). Moreover, *Leishmania* infection also results in compromised production of inflammatory cytokines by infected cells that inhibit differentiation and proliferation of other immune cells involved in its control. In leishmaniasis Th1 phenotypes of CD4 T cells provide resistance by producing inflammatory cytokines such as IL-12, TNF- α whereas IL-10 producing Th2 phenotypes are responsible for disease pathogenesis (Srivastava et al. 2012, Singh et al. 2012). Notwithstanding the mechanisms that controls host immune responses are known but the activators of innate and adaptive immune cells are largely unknown. Studies are required to identify the host/parasite factors to activate effector functions of phagocytic cells especially macrophages since early elimination of parasites boost the establishment of protective immunity. In this study, we report that induced expression of Slc11a1 by host macrophages significantly inhibit *Leishmania* growth and survival. Further, induced Slc11a1 expression was also found correlative to increased production of NO, IL-12 and TNF- α by parasite infected macrophages.

Materials and methods

Leishmania donovani promastigotes and amastigotes culture

The study protocols involving animals for macrophage isolation, and use of *Leishmania* parasites were approved by Animal Ethical Committee of Institute of Science, Banaras Hindu University. The ATCC, USA depository strain of

Leishmania donovani (MHOM/IN/80/DD8) parasites, a gift from Central Drug Research Institute, Lucknow, India, were used in this study. The parasites were maintained in 5–6 week-old female BALB/c mice and promastigotes were cultured from spleen cells. Briefly, animals were sacrificed to remove spleen, which was macerated to obtain homogenate and finally filtered through 45 μ m gauge to recover single cell suspension. Cells were washed twice by centrifugation at 200 rpm for 10 min with serum free Dulbecco's Modified Eagle (DMEM medium). The cells were subsequently cultured in complete DMEM medium (pH 7.2) (DMEM, Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 2 mM L-glutamine, sodium bicarbonate, and antibiotics (Sigma Chemicals, USA); penicillin (100 U/ml), streptomycin (100 μ g/ml), gentamycin (20 μ g/ml) at 26 °C in a BOD incubator to obtain motile promastigote forms of the parasite. Promastigotes were subcultured to get log phase parasites and axenic amastigotes. Briefly, a definite number ($3\text{--}5 \times 10^7$) of promastigotes were taken and washed two times by centrifugation at 3000 rpm for 10 min to remove complete medium. The washed parasites were further cultured in 25 cm² culture flasks in acidified (pH 5.5) complete DMEM medium, and incubated at 37 °C in humidified CO₂ incubator containing 5% CO₂ for their transformation into axenic amastigotes. The amastigotes were characterized by their round or oval shape without flagella and presence of amastigote specific megasomes under phase contrast microscopy. Their biochemical characterization was done by lectin agglutination test (Balanco et al. 1998).

Recovery of amastigote specific proteins

For proteins recovery, amastigotes (2×10^7 /ml) were washed thrice with cold PBS (0.02 M, pH 7.2) by centrifuging at 3000 rpm for 15 min at 4 °C and lysed in lysis buffer containing 20 mM Tris-HCl (pH7.4), 40 mM NaCl, 10 mM EDTA, 2 mM PMSF(Sigma Chemicals, USA), 5 mM iodoacetamide (Sigma, USA), 10 μ g/ml leupeptin (Sigma Chemicals, USA) and 0.4% SDS. Soluble proteins were prepared by sonication (12amp/10cycle/30 s) followed by centrifugation at 10,000 rpm for 20 min. Proteins were resolved on 10% SDS-PAGE and recovered in five protein fractions (Fa, Fb, Fc, Fd, Fe) of definite molecular weights as per standard protocol (Castellanos-Serra et al. 1997).

Isolation of macrophages, quantification of Slc11a1 expression and assessment of parasite burden

Peritoneal macrophages were isolated from female BALB/c mice (average wt, 25 ± 5 g). Briefly, to activate resident peritoneal macrophages, mice were intra peritoneal injected with 1 ml of 2% starch solution. After 5 days, heparin

containing 5 ml cold incomplete DMEM medium was injected in peritoneal cavity and macrophages were drained. Cells were washed twice with PBS by centrifugation at 200 rpm for 5 min and further cultured at 37 °C in humidified condition with 5% CO₂ in a CO₂ incubator (Thermo Scientific, USA). For execution of experiments, macrophages were seeded at a density of 10⁶ cells per ml in 24 well tissue culture plates. After 6 h of activation in presence of various protein fractions, macrophages were washed further incubated for 24 h to quantify Slc11a1 mRNA expression by real time PCR. Total RNA from macrophages was extracted by RNeasy Mini kit (Qiagen cat.74104) following manufacturer instructions. For cDNA preparation, 1 µg total RNA (kept equal in every experiment) was reverse transcribed using 20U MMLV reverse transcriptase (Fermentas, Germany) and 100 ng of random hexamers (Fermentas, Germany). The Slc11a1 expression levels were quantified on ABI 7500 Fast system in a 20 µl reaction mixture containing 10 pmol/µl of Slc11a1 specific primers (F-TGAGCAAAGCCATCACTTCG; R-ATAAG-CAGTCAGGCCCAAGT; product size 149), 10 µl of power SYBER green master mix (Applied Biosystem), 1 µl cDNA and MilliQ water. PCR conditions were set with an initial incubation of 50 °C for 2 min, followed by denaturation at 95 °C for 3 min, and 40 cycles at 95 °C for 45 s, 60 °C for 1 min. The abundance of Slc11a1 mRNA was normalized to geometric average of endogenous control (β-actin; F-ATGGTGGGAATGGGTCAGAA; R-TTGTAGAAGGTGTGGTGCCA) for ΔCt. The fold change (ΔCt) was calculated as the difference between experimental groups versus naïve non-infected macrophages. For assessment of parasite burden, proteins activated and non-activated macrophages were infected with *L. donovani* promastigotes in parasite to cell ratio of 10:1 for 6 h and subsequently washed to remove non-ingested parasites and kept in CO₂ incubator for the desired duration. The parasite burden (intracellular amastigotes) was counted by Geimsa staining after 24 and 48 h.

Measurement of superoxide anion (O₂^{•-}), H₂O₂, NOx and cytokines levels

The superoxide anion content was estimated by the method described elsewhere (Chakraborty et al. 1996) and expressed nmoles of O₂^{•-} liberated per mg of protein. The production of hydrogen peroxide was measured fluorometrically according to the method described elsewhere (Singh et al. 2013). The H₂O₂ generated was estimated using standard curve pre-calibrated with known amount of H₂O₂ and represented as nmoles per 10⁶ cells. Total nitric oxide production was assayed in culture supernatant by Griess reagent as described elsewhere (Ghasemi et al. 2008). The extracellular cytokines levels for TNF-α, IL-12 and IFN-γ were detected by ELISA

MAXTM standard set enzyme-linked immunosorbent assay kit as per manufacture instructions (Biolegend, USA) in culture supernatants. The results were represented in pg of cytokines/ml. The O₂^{•-}, H₂O₂ were quantified 2 h post infection, NOx at 24 h and cytokines were estimated cultured supernatant 78 h post infection.

Measurement of labile iron pool (LIP) status

In cytosolic compartment of the cells, the labile iron pool (LIP) is redox-active chelatable iron that can be quantified by fluorescent iron probes (Cabantchik et al. 1996). Calcein-AM (Sigma Chemicals, USA), a membrane permeable dye, is cleaved by non-specific esterases of macrophages into free fluorescent calcein and non-fluorescent acetomethoxy group in macrophage cytoplasm. Calcein immediately binds with chelatable iron ions that result in quenched fluorescence. Briefly, after 24 and 48 h incubation of infected and non-infected macrophages, hemin (Sigma Chemicals, USA) at a concentration of 500 µM was added to the culture medium and further incubated for 45 min. After washing with PBS, cells were suspended in 2 ml PBS in a polypropylene microfuge tube and incubated with calcein-AM (0.125 µM) for 20 min at RT. After incubation, cells were washed to remove extracellular calcein, re-suspended in 2 ml PBS, and fluorescence was measured at excitation wavelength 488 nm and emission wavelength 517 nm. After 2 min interval, salicylaldehyde isonicotinoyl hydrazone (SIH) was added at a concentration of 200 µM. SIH is a strong iron chelator and has more affinity for iron than calcein. It chelates calcein bound iron and releases free calcein that dequench the fluorescence. After 5 min of SIH addition fluorescence was again measured. The difference in the fluorescent intensity between before and after addition of SIH was proportional to the amount of LIP. The LIP was expressed in terms of arbitrary units of fluorescent intensity ΔF (AU) = fluorescent intensity after SIH addition—fluorescent intensity before SIH addition).

Results

Amastigotes specific proteins significantly induced Slc11a1 mRNA expression

The silver staining of SDS-PAGE revealed the presence of several proteins ranging from 10 to 170 kDa in crude soluble extract of amastigotes is shown in Fig. 1a. On the basis of relative molecular weights, protein were divide in five fractions viz. Fa (170–73 kDa), Fb (72–43 kDa), Fc (42–27 kDa), Fd (26–18 kDa) and Fe (17–10 kDa) and purified after recovery. To identify their potential as Slc11a1 inducer, macrophages were activated (10 µg/ml)

Fig. 1 **a** A silver stained 10% SDS-PAGE gel showing various crude soluble proteins (CSP) of *L. donovani* amastigotes ranging from 10 to 170 kDa [M: mw markers, A: CSPs (40 µg/well)]. Based on their relative mw, proteins were divided in five fractions (Fa, Fb, Fc, Fd, Fe), eluted and purified before use in macrophage activation. **b** the Slc11a1 mRNA expression levels in proteins activated non-infected macrophages. Macrophages were activated by fractions protein (10 µg/ml) for 6 h and after subsequent washing further incubated for 24 h in CO₂ incubator. All quantifications were normalized to β-actin as a house keeping gene, and expression levels are represented in comparative fold change in proteins activated macrophages and non-activated control. A significant ($p < 0.001$) increase in mRNA expression was observed in Fb activated macrophages whereas Fd induced least expression

with these fractions and Slc11a1 mRNAs were quantified after 24 h post incubation. On comparison of five fractions, Slc11a1 expression was observed significantly ($p < 0.001$) high in Fb activated cells and Fd fraction induced least expression (Fig. 1b).

Induced SLC11A1 expression significantly inhibited parasites growth with increased LIP status in Fb proteins activated macrophages

To identify Slc11a1 role in *Leishmania* control, we selected fraction Fb and Fd as high and low inducers of Slc11a1 expression for a comparative analysis. After activation by protein fractions, macrophages were infected with *L. donovani* promastigotes and incubated for 24 and 48 h. The clearance of parasites in infected cells was found highly correlative to increased Slc11a1 expression. Fb fraction resulted in more significant inhibition of parasite survival in infected macrophages (FbAIM) at both, 24 and 48 h ($p < 0.01$) post infection compared non-activated infected macrophages (NAIM). The parasite growth was not found inhibited in Fd induced macrophages (FdAIM) and was found comparable to NAIM at both time points (Fig. 2a). This showed that increased Slc11a1 expression results in efficient killing of intracellular parasites. To confirm functioning of this pump, we assessed LIP status of parasite infected macrophages as it directly inform about increased iron in cell cytoplasm. The labile iron pool (LIP) is weakly bound redox-active chelatable iron in cytosolic compartment, which is tightly regulated but easily accessible for

Fig. 2 Parasite load and LIP status in proteins activated and non-activated macrophages. Proteins fraction Fb and Fd were used as high and low Slc11a1 inducer for comparative study. Macrophages were first activated for 6 h and washed. Macrophages were infected to parasites in ratio of 10:1 (parasite: cell) for 6 h, and subsequently washed to remove non-internalized parasites. **a**: The intracellular amastigotes were counted by Geimsa staining and represented as average number of amastigotes per hundred infected macrophages. Fb fraction resulted in significant inhibition of parasite growth at both, 24 h and 48 h ($a = p < 0.01$ vs. FdAIM & NAIM) as compared to NAIM whereas it was not found inhibited in FdAIM. [FbAIM: Fb protein activated infected macrophages, FdAIM: Fb protein activated infected macrophages, NAIM: non-activated infected macrophages]. **b** The LIP status of macrophage cytoplasm was found significantly elevated in FbAIM ($a = p < 0.01$ vs. FdAIM & NAIM) whereas in FdAIM, it was comparable to NAIM

cellular metabolic activities. The LIP status was also found highly correlative to increased Slc11a1 expression as we observed a significant increase in cytoplasmic labile iron pool in Fb activated cells at both, 24 and 48 h post infection ($p < 0.01$ vs. NAIM) whereas in FdAIM it was comparable to NAIM (Fig. 2b). For a comparison and assure Slc11a1 expression in infected macrophages, we also quantified Slc11a1 expression in proteins activated and non-activated infected macrophages at 24 h post infection and it was observed maximum in FbAIM group (data not shown).

Slc11a1 functionality was correlated with increased production of NO and IL-12 in Fb proteins activated macrophages

We did not observe any significant difference in levels of super oxide anions and H₂O₂ in proteins activated and non-activated macrophages (Fig. 3). However, the level of

Fig. 3 **a** The levels of $O_2^{\cdot-}$, H_2O_2 , and total NOx generation by activated and non-activated parasite infected macrophages, which are expressed in nmoles/mg of protein, nmoles/10⁶ cells and μM, respectively. The generation of super oxide anions and H_2O_2 in proteins activated and non-activated macrophages was observed statistically similar. The NO level was found increased in Fb activated macrophages (a = $p < 0.05$ vs. FdAIM; b = $p < 0.01$ vs. NAIM) **b** Levels of IFNγ, TNFα and IL-12 release by *Leishmania* infected macrophages in activated and non-activated cells post 72 h of incubation. The levels of IL-12 (a = $p < 0.01$ vs. FdAIM; b = $p < 0.05$ vs. NAIM) and TNFα (a = $p < 0.01$ vs. FdAIM and NAIM) were increased in Fb proteins activated macrophages whereas IFNγ levels were statistically similar in proteins activated and non-activated macrophages

nitric oxide was found increased ($p < 0.05$ vs. FdAIM; $p < 0.01$ vs. NAIM) in Fb activated macrophages post 24 h infection. The Th1 cytokines generation by activated and non-activated macrophages was quantified in terms of IFNγ, TNFα and IL-12. Fb activation resulted in increased production of TNFα ($p < 0.01$ vs. FdAIM & NAIM) and IL-12 ($p < 0.01$ vs. FdAIM; $p < 0.05$ vs. NAIM) whereas IFNγ levels were statistically similar in proteins activated and non-activated macrophages.

Discussion and conclusion

VL infections are endemic in various part of the world including Indian subcontinent where disease prevalence is very high. The global estimates indicate about 100,000 VL cases per year out of which 15,000 cases are being reported each year (WHO 2015). In Indian subcontinent, India contributes more than 80% of reported cases followed by Bangladesh, Nepal, Thailand and Bhutan (WHO 2005). The parasites have developed resistance against the only

true antileishmanial pentavalent antimony compounds in disease endemic regions (Chakravarty and Sundar 2010). Leishmanial infections are currently being treated by either amphotericin B or miltefosine (Singh et al. 2012), which are potentially toxic and produce serious side effects (Freitas-Junior et al. 2012). Unfortunately, in recent years resistance is being reported in clinical settings against both drugs that strongly necessitates the search for alternate control strategies (Purkait et al. 2012). It is also believed that these drugs do not produce sterile cure, which is a serious limitation because such cured patients act as a parasite carrier and may spread diseases in endemic regions (Prajapati et al. 2012). Further, the parameters of protective immunity are not well defined or identified in VL pathogenesis, which is a major hurdle in development of vaccine. These lacunas necessitate identification of host/parasite factors not only to activate host immunity but also to directly target parasites. The findings of present study suggest that activation of Slc11a1 can be efficiently used to control survival and proliferation of *Leishmania* within infected host macrophages. Additionally, the induced Slc11a1 expression was also found highly correlated with generation of inflammatory response in infected macrophages further suggest Slc11a1 role in activation innate immunity.

Expression of Slc11a1 has been found very decisive for host resistance against invading pathogens as it creates cations deprived condition in phagolysosomes, which are required for survival of intracellular parasites (Huynh and Andrews 2008). Inside the host macrophages, *Leishmania* essentially requires Fe^{2+} along with other divalent cations for its enzymatic machinery, which are involved in growth, survival and virulence. Studies have revealed that *Leishmania* acquires iron by depleting labile iron pool by modulating either transferring receptor expression for increased uptake of TfR bound iron and induced expression of LIT-1 iron transporter on its surface (Das et al. 2009). However, the mammalian defense system has also evolved several ways to deprive microorganisms for these vital elements in order to curb parasitic growth. As a cationic transporter, Slc11a1 pumps out iron from phagolysosomal milieu to cytosolic compartment of macrophages as a host natural strategy to kill intracellular parasites (Jabado et al. 2000). In Fb activated macrophages, parasite growth was significantly inhibited that was found linked with LIP status in macrophages cytoplasm. This finding suggested the increased functionality of Slc11a1 ensures low availability of iron in phagolysosomes by pumping them out in cell cytoplasm. In addition, the levels of NO and inflammatory cytokines were also found significantly higher in Fb protein activated macrophages, which further supported the fact that increases Slc11a1 functionality helps macrophages to sustain its effector properties. Slc11a1 expression has also

been shown to promote Fpn1 expression, an exclusive iron exporter of macrophages, to maintain optimal level of iron in cell cytoplasm that further ensure minimum availability of iron to intracellular parasites (Soe-Lin et al. 2008). Therefore, these findings provide strong evidence on the role of this pump in *Leishmania* killing. Other studies have also provided an efficient role of this pump to control pathogens growth and survival. In *Salmonella* species, activated Slc11a1 expression by lipocalin-2 has shown to control their growth through impaired iron acquisition and induction of macrophage effector function (Nairz et al. 2009, Fritsche et al. 2012).

Innate immune response also play very important role in early parasite elimination and help in development of long lasting protective immunity for disease resistance (Gupta et al. 2013). The survival of parasites within infected macrophages is determined by the magnitude of its activation by host and parasitic factors. The parasite evades killing by activated macrophages and guide them to produce more immune inhibitory cytokines such as IL-10 and TGF- β for survival and proliferation (Kane and Mosser 2001). In VL, generation of inflammatory IL-12 by macrophages protects host through development of protective Th1 type immune response (Liu and Uzonon 2012, Gupta et al. 2013). Further, early clearance of *Leishmania* by host phagocytic cells also produce long lasting protective immunity that further suggest importance of efficient macrophage function in VL control (Selvapandiyan et al. 2009). Since Slc11a1 overexpression was found correlated to induced production of IL-12 in Fb proteins activated macrophages, it may be hypothesized that Slc11a1 functionality is required to activate infected macrophages. Studies have shown that Slc11a1 also regulates macrophages redox status via induction of inflammatory cytokines and NO levels via induced iNOS expression along with suppressed IL-10 production (Fritsche et al. 2008, Singh et al. 2013). We also observed comparatively more NO and IL-12 levels in Fb activated cells, which can be an additional factor for reduced parasitic survivals since NO is a major effectors molecule for anti-leishmanial activity (Dempsey 2013). Moreover, in VL infected individuals the disease resistance is conferred by induction of Th1 type immune response, which suggests the potential of amastigote specific proteins to elicit protective immune response (Nylen and Gautam 2010). The identified Fb fraction proteins also suggest such possibility but require further elucidation and identification of antigenic fractions. In nutshell, this study provides a ground that Slc11a1 inducing leishmanial proteins can augment protective innate immune response, which can also be exploited to find a vaccine candidate. Although, the stimulation of macrophages with identified leishmanial proteins significantly restricted growth and survival of intracellular

amastigotes but it requires more studies to identify the candidate protein/s and delineation of Slc11a1 associated mechanisms in control of *Leishmania donovani* infection.

Acknowledgements Financial support from Department of Science and Technology, New Delhi (SB/SO/HS/0091/2013) is greatly acknowledged. The authors MGR and NT are extremely thankful to BHU and UGC, New Delhi, respectively for their research fellowships.

References

- Appelberg R (2006) Macrophage nutritive antimicrobial mechanisms. *J Leukoc Biol* 79:1117–1128
- Balanco JM, Pral EM, da Silva S, Bijovsky AT, Mortara RA, Alfieri SC et al (1998) Axenic cultivation and partial characterization of *Leishmania braziliensis* amastigote-like stages. *Parasitology* 116:103–113
- Blackwell JM, Searle S, Mohamed H, White JK (2003) Divalent cation transport and susceptibility to infectious and autoimmune disease: continuation of the Ity/Lsh/Bcg/Nramp1/Slc11a1 gene story. *Immunol Lett* 85:197–203
- Cabantchik ZI, Glickstein H, Milgram P, Breurer W (1996) A fluorescence assay for assessing chelation of intracellular iron in a membrane model system and in mammalian cells. *Anal Biochem* 233:221–227
- Caron J, Lariviere L, Nacache M, Tam M, Stevenson MM, McKerly C et al (2006) Influence of Slc11a1 on the outcome of *Salmonella enterica* serovar Enteritidis infection in mice is associated with Th polarization. *Infect Immun* 74:2787–2802
- Castellanos-Serra LR, Fernandez-Patron C, Hardy E, Santana H, Huerta V (1997) High yield elution of proteins from sodium dodecyl sulfate-polyacrylamide gels at the low-picomole level. Application to N-terminal sequencing of a scarce protein and to in-solution biological activity analysis of on-gel renatured proteins. *J Protein Chem* 16:415–419
- Cellier MF, Courville P, Campion C (2007) Nramp1 phagocyte intracellular metal withdrawal defense. *Microbes Infect* 9:1662–1670
- Chakraborty R, Mukherjee S, Basu MK (1996) Oxygen-dependent leishmanicidal activity of stimulated macrophages. *Mol Cell Biochem* 154:23–29
- Chakravarty J, Sundar S (2010) Drug resistance in leishmaniasis. *J Glob Infect Dis* 2:167–176
- Dai YD, Marrero IG, Gros P, Zaghoulani H, Wicker LS, Sercarz EE (2009) Slc11a1 enhances the autoimmune diabetogenic T-cell response by altering processing and presentation of pancreatic islet antigens. *Diabetes* 58:156–164
- Das NK, Biswas S, Solanki S, Mukhopadhyay CK (2009) *Leishmania donovani* depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. *Cell Microbiol* 11:83–94
- Dempsey LA (2013) Metabolic control of cytokines. *Nat Immunol* 14:776
- Forbes JR, Gros P (2001) Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends Microbiol* 9:397–403
- Freitas-Junior LH, Chatelain E, Kim HA, Siqueira-Neto JL (2012) Visceral leishmaniasis treatment: what do we have, what do we need and how to deliver it? *Int J Parasitol Drugs Drug Resist* 2:11–19
- Fritsche G, Dlaska M, Barton H, Theurl I, Garimorth K, Weiss G (2003) Nramp1 functionality increases inducible nitric oxide

- synthase transcription via stimulation of IFN regulatory factor 1 expression. *J Immunol* 171:1994–1998
- Fritsche G, Nairz M, Theurl I, Mair S, Bellmann-Weiler R, Barton HC et al (2007) Modulation of macrophage iron transport by Nramp1 (Slc11a1). *Immunobiology* 212:751–757
- Fritsche G, Nairz M, Werner ER, Barton HC, Weiss G (2008) Nramp1-functionality increases iNOS expression via repression of IL-10 formation. *Eur J Immunol* 38:3060–3067
- Fritsche G, Nairz M, Libby SJ, Fang FC, Weiss G (2012) Slc11a1 (Nramp1) impairs growth of *Salmonella enterica* serovar *typhimurium* in macrophages via stimulation of lipocalin-2 expression. *J Leukoc Biol* 92:353–359
- Ghasemi A, Zahedi Asl S, Mehrabi Y, Saadat N, Azizi F (2008) Serum nitric oxide metabolite levels in a general healthy population: relation to sex and age. *Life Sci* 83:326–331
- Gruenheid S, Cellier M, Vidal S, Gros P (1995) Identification and characterization of a second mouse Nramp gene. *Genomics* 25:514–525
- Gupta G, Oghumu S, Satoskar AR (2013) Mechanisms of immune evasion in leishmaniasis. *Adv Appl Microbiol* 82:155–184
- Huynh C, Andrews NW (2008) Iron acquisition within host cells and the pathogenicity of *Leishmania*. *Cell Microbiol* 10:293–300
- Jabado N, Jankowski A, Dougaparsad S, Picard V, Grinstein S, 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
- Kane MM, Mosser DM (2001) The role of IL-10 in promoting disease progression in leishmaniasis. *J Immunol* 166:1141–1147
- Liese J, Schleicher U, Bogdan C (2008) The innate immune response against *Leishmania* parasites. *Immunobiology* 213:377–387
- Liu D, Uzonna JE (2012) The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect Microbiol* 2:83
- Nairz M, Fritsche G, Crouch ML, Barton HC, Fang FC, Weiss G (2009) Slc11a1 limits intracellular growth of *Salmonella enterica* sv. *Typhimurium* by promoting macrophage immune effector functions and impairing bacterial iron acquisition. *Cell Microbiol* 11:1365–1381
- Nylen S, Gautam S (2010) Immunological perspectives of leishmaniasis. *J Glob Infect Dis* 2:135–146
- Prajapati VK, Awasthi K, Yadav TP, Rai M, Srivastava ON, Sundar S (2012) An oral formulation of amphotericin B attached to functionalized carbon nanotubes is an effective treatment for experimental visceral leishmaniasis. *J Infect Dis* 205:333–336
- Purkait B, Kumar A, Nandi N, Sardar AH, Das S, Kumar S et al (2012) Mechanism of amphotericin B resistance in clinical isolates of *Leishmania donovani*. *Antimicrob Agents Chemother* 56:1031–1041
- Selvapandiyan A, Dey R, Nylen S, Duncan R, Sacks D, Nakhasi HL (2009) Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. *J Immunol* 183:1813–1820
- Singh N, Kumar M, Singh RK (2012) Leishmaniasis: current status of available drugs and new potential drug targets. *Asian Pac J Trop Med* 5:485–497
- Singh N, Bajpai S, Kumar V, Gour JK, Singh RK (2013) Identification and functional characterization of *Leishmania donovani* secretory peroxidase: delineating its role in NRAMP1 regulation. *PLoS ONE* 8:e53442
- Soe-Lin S, Sheftel AD, Wasyluk B, Ponka P (2008) Nramp1 equips macrophages for efficient iron recycling. *Exp Hematol* 36:929–937
- Srivastava A, Singh N, Mishra M, Kumar V, Gour JK, Bajpai S et al (2012) Identification of TLR inducing Th1-responsive *Leishmania donovani* amastigote-specific antigens. *Mol Cell Biochem* 359:359–368
- Stober CB, Brode S, White JK, Popoff JF, Blackwell JM (2007) Slc11a1, formerly Nramp1, is expressed in dendritic cells and influences major histocompatibility complex class II expression and antigen-presenting cell function. *Infect Immun* 75:5059–5067
- Valdez Y, Diehl GE, Vallance BA, Grassl GA, Guttman JA, Brown NF et al (2008) Nramp1 expression by dendritic cells modulates inflammatory responses during *Salmonella Typhimurium* infection. *Cell Microbiol* 10:1646–1661
- Weiss G, Werner-Felmayer G, Werner ER, Grunewald K, Wachter H, Hentze MW (1994) Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J Exp Med* 180:969–976
- WHO (2005) Regional Strategic Framework for Elimination of Kala-azar from the South-East Asia Region (2005–2015). New Delhi
- WHO (2015) Kala-azar elimination programme. Switzerland, Geneva