NRAMP-1 Expression Modulates Protein-tyrosine Phosphatase Activity in Macrophages

IMPACT ON HOST CELL SIGNALING AND FUNCTIONS*

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NRAMP-1 (natural resistance-associated macrophage protein-1) has been associated with innate resistance to unrelated intracellular pathogen infections, up-regulation of proinflammatory phagocyte functions, and susceptibility to autoimmune diseases. It is still unclear how the divergent cation transport function of NRAMP-1 accounts for the associated pleiotropic effects. In this study, we evaluated the impact of murine macrophage NRAMP-1 expression on the activity of protein-tyrosine phosphatases (PTPs) as an upstream event contributing to the NRAMP-1 regulation of signal transduction and control of effector macrophage functions. Functional expression of NRAMP-1 results in lower macrophage PTP activity and increased protein phosphorylation. Decreased PTP activity is not a result of changes in protein expression but rather a reversible regulatory mechanism involving the interaction with NRAMP-1 metal substrates. In the context of intracellular infections, NRAMP-1 expression prevents full macrophage PTP induction by Leishmania infection, correlating with higher nitric oxide production and lower parasite survival. We suggest that NRAMP-1 divalent cation transport leads to transient inhibition of PTPs via direct PTP-metal interaction and/or by reactive oxygen species-dependent PTP oxidation, consequently promoting positive signal transduction, as a backbone for the induction of proinflammatory phagocyte functions.

NRAMP-1 (natural resistance-associated macrophage protein-1), previously known as Bcg/Ity/Lsh, and recently renamed Slc11a1) has been associated with host resistance to unrelated intracellular pathogens (1, 2), including Leishmania (3, 4), Mycobacterium (5), and Salmonella (6). It has also been associated with the up-regulation of proinflammatory macrophage (Mφ)3 functions, such as major histocompatibility complex II expression (7, 8), KC chemokine (9, 10), interleukin-1β (9), tumor necrosis factor-α (11, 12), nitric oxide (NO) production (13, 14), and increased respiratory burst (15, 16). NRAMP-1 is a pH-dependent divalent cation transporter (17) localized to the late endosome/lysosomal compartment of Mφs from the reticuloendothelial system (18) and present in gelatinase-positive tertiary granules of neutrophils (19). Upon phagocytosis, NRAMP-1 is rapidly recruited to the phagolysosomal membrane, where it mediates the transport of Mn2+, Fe2+, Co2+, and potentially other metals, including Zn2+ (20–23). The direction of metal flux is still controversial (17, 24, 25); however, transport studies, sequence and structural similarities with NRAMP-2, topology and thermodynamic considerations suggest that metal transport occurs from the vesicular lumen to the cytoplasm (17). NRAMP-1 expression has been shown to promote phagosome maturation (26–28). In addition, efflux of essential metals from the phagosome may restrain the pathogen’s development by interfering with essential microbial enzymes, such as superoxide dismutase and/or by promoting the up-regulation of host proinflammatory molecules. Although more than 10 years have passed since the positional cloning and characterization of NRAMP-1 (1, 29), it still remains unclear how the divergent cation transport function of this protein contributes to and mediates the multiple pleiotropic events associated with its functional expression.

Protein phosphorylation plays a fundamental role in signaling pathways. The fine balance of protein kinase and protein phosphatase activities promotes the homeostasis of protein phosphorylation. Alterations in this balance, however, permit the unfolding of signal transduction. It has been previously shown that NRAMP-1-expressing Mφs have a higher protein kinase C activity compared with NRAMP-1-deficient cells. This was associated with the induction of NO production, respiratory burst, and NRAMP-1 mRNA stability (30, 31). Having formerly observed that modulation of protein-tyrosine phospha-
tases (PTP) greatly influences signaling and phagocyte functions (32–35), we sought to determine whether NRAMP-1 expression impacts PTP activity as a mechanism for regulating signaling pathways and downstream cellular events. Results from the present study reveal that NRAMP-1 expression has an effect on Mφ PTP activity, in turn modulating the phosphorylation/activation of signaling proteins associated with LPS- and IFN-γ-dependent NO production and the Mφ response to Leishmania infection. Our data and ongoing research suggests that the mechanism underlying this regulation involves a metal-dependent reversible inhibition of PTP activity.

EXPERIMENTAL PROCEDURES

Materials—4-Nitrophenylphosphate disodium salt hexahydrate (pNPP), LPS (Escherichia coli, serotype 0111:B4), catalase, superoxide dismutase, poly(Glu,Tyr), phosphotyrosine peptide substrate (TRDiPYETDYRRK), N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), and deferoxamine (DFO) were purchased from Sigma. [γ-32P]dATP (3000 Ci/mmole) was obtained from GE Healthcare. Guanidine hydrochloride was purchased from Laboratoire MAT (Beauport, Canada). Recombinant IFN-γ was obtained from Cedarlane Laboratories.

Cell Culture—RAW 264.7 murine Mφ cell line was kept in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 2 mM L-glutamine at 37 °C and 5% CO2.Nramp-1-transfected RAW 264.7 (RAW Nramp-1) were maintained in selective Dulbecco's modified Eagle's medium containing 1 mg/ml G418 (36). Leishmania donovani strain stably transfected with the luciferase reporter gene were kept in SDM-79 medium at 25 °C supplemented with 10% heat-inactivated fetal bovine serum and 5 mg/ml of G418 (36).

In Vitro Infections—Stationary phase Ld promastigotes were used to infect Mφs for 1 h at 37 °C in a 20:1 Ld-Mφ ratio (unless noted). Noninternalized parasites were removed by washing the plates with phosphate-buffered saline (PBS), after which Mφs were collected for subsequent procedures. For parasite survival determinations, Mφs were infected with a 10:1 Ld-Mφ ratio for 6 h, washed with PBS, and followed by a chase period of 24 h. Parasite survival was estimated by determining luciferase activity (38).

Phosphatase Assays—As previously described (39), infected and noninfected Mφs were collected, lysed in PTP lysis buffer (50 mM Tris, pH 7.0, 0.1 M EDTA, 0.1 M EGTA, 0.1% 2-mercaptoethanol, 1% Igepal, 25 μg/ml aprotinin, and 25 μg/ml leupeptin) and kept on ice for 45 min. Lysates were cleared by centrifugation, and protein content was determined by Bradford's method (40). Ten μg of protein extract were incubated in phosphatase reaction buffer (50 mM Hepes, pH 7.5, 0.1% 2-mercaptoethanol, 10 mM pNPP) for 30 min. OD was read at 405 nm. Specific PTP activity was determined by the capacity of protein lysates to dephosphorylate a monophosphorylated phosphotyrosine peptide substrate (TRDiPYETDYRRK) for 10 min at 37 °C. Free inorganic phosphate was detected with malachite green (Sigma), and OD was taken at 620 nm.

In-gel PTP Assay—An in-gel PTP assay was performed as previously described (41, 42). Briefly, poly(Glu,Tyr) substrate was tyrosine-phosphorylated by overnight (ON) incubation with 10 μg of purified glutathione S-transferase-FER protein kinase and 150 μCi of [γ-32P]dATP. The radiolabeled phosphorylated substrate was incorporated in a 10–12% SDS-polyacrylamide gel mixture at a concentration of 2 × 105 cpm/ml of gel solution. Mφ protein extracts were prepared as described above, denatured by standard SDS-PAGE procedures, and loaded onto the gel. After electrophoresis, the gel was incubated ON in Buffer A (50 mM Tris-HCl, pH 8.0, 20% isopropyl alcohol). Gels were washed twice with Buffer B (50 mM Tris-HCl, pH 8.0, 0.3% β-mercaptoethanol), followed by full protein denaturation in Buffer B containing 6 M guanidine hydrochloride and 1 mM EDTA. The gels were washed twice in Buffer C (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.3% β-mercaptoethanol, and 0.04% Tween 20). Final renaturation was obtained after an ON incubation in Buffer C. Gels were dried and exposed to x-ray film.

Western Blotting—Western blots were performed as previously described (43). Primary antibodies used were α-phosphotyrosine clone 4G10, α-SHP-1, and α-PTP1B (Upstate Biotechnology, Inc., Lake Placid, NY); α-SHP-2 and α-STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA); α-phosphotyrosine STAT1 (Tyr701) (Cell Signaling Technologies); and α-phospho-ERK1/2 (Tyr202/Tyr204), α-ERK1/2, α-phospho-MEK1/2 (Ser217/221), and α-MEK1/2 (New England Biolabs). α-TCPTP 3E2 (44) and α-PTP-PEST 2530 (45) were obtained as described.

Electrophoretic Mobility Shift Assay—An electrophoretic mobility shift assay was performed as described before (43). Briefly, nuclear extracts were incubated with binding buffer containing 1.0 ng of [γ-32P]dATP radiolabeled double-stranded DNA oligonucleotide for 20 min at room temperature. The oligonucleotides used were the DNA binding consensus sequences for NF-κB (5'-AGTTGAGGGACTTCCCAGGC-3') and GAS/ISRE (5'-AAGTACTTTCATTTATTCTA-3'). Sp1 consensus oligonucleotide was used as unspecific control (5'-ATTCGATCGGGGCGAGC-3') (Santa Cruz Biotechnology). DNA-protein complexes were resolved by electrophoresis in native 4% (w/v) polyacrylamide gels. The gels were subsequently dried and autoradiographed.

Confocal Microscopy—Cells were plated ON in glass cover slips, washed with cold PBS, fixed with 4% formaldehyde at 4 °C, and permeabilized for 5 min in PBS containing 1% bovine serum albumin and 0.05% Nonidet P-40. After blocking in 5% nonfat evaporated milk in PBS, the coverslips were incubated with α-phosphotyrosine antibody. Slides were washed with PBS incubated with AlexaFluor 488 α-mouse antibody (Molecular Probes, Inc., Eugene, OR). Nuclear stain was performed with propidium iodide. After mounting, cells were visualized by confocal microscopy (×63), using a Zeiss LSM 510 system.

NO Production—Mφs were seeded in 12-well plates and cultured in the presence or absence of purified E. coli LPS for 24 h or IFN-γ for 48 h. NO production was evaluated by measuring the accumulation of nitrite in the culture medium by the Griess reaction, as previously described (46).
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### Results

**NRAMP-1 Regulation of NO Production Is Associated with Modulation of Signaling Pathways**—One of the major Mφ functions associated with NRAMP-1 expression and the successful control of intracellular pathogens is NO production (13, 14). By using the Mφ cell line RAW 264.7 (naturally deficient in a functional NRAMP-1) and NRAMP-1-transfected RAW cells (RAW Nramp-1) (36), we have studied the signaling pathways underlying IFN-γ- and LPS-dependent NO production as a means to functionally validate the resistance phenotype of our Mφ cell line system.

LPS stimulation induced a higher and dose-dependent (2–10-fold) nitrite accumulation in RAW Nramp-1 cells, compared with its NRAMP-1-deficient counterpart (Fig. 1A). As with LPS, NO production following IFN-γ stimulation was significantly higher in RAW Nramp-1 cells (Fig. 1D). Previous reports have shown that IFN-γ- and LPS-dependent NO production in NRAMP-1-expressing Mφs results from increased iNOS mRNA stability and transcription (14). Evaluation of MEK/ERK and JAK/STAT signaling pathways, responsible for the induction of iNOS expression (47–49), showed that LPS and IFN-γ strongly induced the respective phosphorylation and activation of MEK1/2, ERK1/2 (Fig. 1B), and STAT1 (Fig. 1E) in RAW Nramp-1 cells at all time points during a 1-h time course stimulation, resulting in increased and sustained nuclear translocation and DNA binding activity of NF-κB (Fig. 1C) and STAT1 (Fig. 1F). These results are in support of previous observations (50, 51) validating our *in vitro* system.

**Macrophage PTP Activity Is Affected by the Functional Expression of NRAMP-1**—In order to understand the mechanism by which NRAMP-1 mediates the regulation of protein phosphorylation and activation of signaling pathways, we studied its impact on the direct coordinators of tyrosine phosphorylation. Protein-tyrosine phosphorylation is tightly controlled by the balanced activity of protein-tyrosine kinases and PTPs. PTPs, comprising the largest gene family of phosphatases in the human genome (52, 53), are key regulators of signal transduction in multiple cellular processes, including differentiation, activation, and response to infection. Basal Mφ PTP activity was determined by the capacity of total Mφ protein lysates to dephosphorylate the phosphate analogue pNPP and a synthetic tyrosine monophosphorylated peptide substrate. Dephosphorylation of the two PTP substrates showed a significant 35% reduction of PTP activity in response to NRAMP-1 expression (Fig. 2A), contributing to the higher phosphorylation levels of some phosphoproteins, including STAT1, ERK1/2, and MEK1/2, and increased kinase activities (31, 51). To evaluate the effect of the differential PTP activity, tyrosine phosphorylation of Mφ proteins from both cell lines was monitored by Western blot analysis of protein lysates following LPS or IFN-γ stimulation (Fig. 2B). These experiments showed increased and sustained phosphorylation of the two protein substrates following LPS or IFN-γ stimulation in RAW Nramp-1 cells, compared with its NRAMP-1-deficient counterpart (Fig. 2B). These results are in support of previous observations (50, 51) validating our *in vitro* system.

**Statistical Analysis**—Data were analyzed by one-way analysis of variance. Difference between groups was considered statistically significant when *p* was <0.05 or <0.01. All data are presented as the means ± S.E.

**FIGURE 1.** NRAMP-1 expression modulates NO production by regulating the phosphorylation of signaling proteins. Mφs were stimulated with 10–100 ng/ml LPS (A) or 500–1000 units/ml IFN-γ (D) for 24 h. NO production was evaluated by quantification of nitrite accumulation in the supernatant of cultured Mφs using a Griess reaction (46). MEK1/2, ERK1/2 (B), and STAT1 (E) phosphorylation was evaluated by Western blot (WB) of total Mφ protein lysates following 0–60 min stimulation with LPS (50 ng/ml) or IFN-γ (500 units/ml). Shown are the loading controls using anti-MEK1/2, anti-ERK1/2, and anti-STAT1 antibodies. Kinetic studies (0–2 h) of the translocation and DNA binding activity of the transcription factors NF-κB (C) and STAT1 (F) following LPS or IFN-γ stimulation were performed by electrophoretic mobility shift assay analysis of nuclear extracts. Data are representative of three individual experiments (B, C, E, and F) or mean values of three independent experiments performed in duplicate ± S.E. (A and D). Differences were considered statistically significant (**) when *p* was <0.05. *Sp*, specific competitor. *NS*, nonspecific competitor.
phosphorylation at basal level and upon latex beads phagocytosis (data not shown). Furthermore, anti-phosphotyrosine Western blot showed increased phosphorylation in RAW Nramp-1 protein bands ranging from 30 to 150 kDa (Fig. 2C), suggesting that inhibition of more than one PTP is involved in the maintenance of higher protein phosphorylation.

Modulation of PTP Activity in NRAMP-1-deficient M$$\phi$$s Reverts Their Functional Phenotype—Peroxovanadium-derived compounds have been shown by our laboratory (35) and others (54) to be specific and potent PTP inhibitors that modulate phagocyte functions \textit{in vitro} and \textit{in vivo}. To demonstrate the contribution of differential PTP activity in the signaling and functional events associated with NRAMP-1 expression, RAW and RAW Nramp-1 cells were incubated with the peroxovanadium-derived compound bpV(phen) to reach equivalent PTP activity levels in both cell lines. As seen in Fig. 3A, treatment with doses ranging from 5 to 25 \(\mu\text{M}\) bpV(phen) result in equivalent PTP activity for RAW and RAW Nramp-1 M$$\phi$$s. Interestingly, 1 h of bpV(phen) treatment prior to LPS stimulation promotes similar and enhanced capacity of both cell lines to produce NO (Fig. 3B). Moreover, inhibition of PTP activity in RAW M$$\phi$$s even with the lower dose of bpV(phen) (5 \(\mu\text{M}\)) rescues the functional phenotype and induces NO production to the same level of bpV(phen)-untreated RAW Nramp-1 M$$\phi$$s. This finding suggests that the basal difference in PTP activity is crucial for the differential regulation of NO given that equivalent levels of PTP activity in both cell lines, achieved by partial inhibition with bpV(phen), rescue the capacity of NRAMP-1-deficient M$$\phi$$s to produce NO.

NRAMP-1 Expression Prevents Full Induction of M$$\phi$$ PTP Activity upon Leishmania Infection—As previously shown by our group, Leishmania infection strongly induces M$$\phi$$ PTP activity, particularly that of the Src homology 2 domain containing PTP SHP-1, as a mechanisms to down-regulate M$$\phi$$ functions and promote successful intracellular survival (32–35, 39, 46). In light of the observation that NRAMP-1 expression plays a role in the regulation of M$$\phi$$ PTP activity, we sought to determine if the difference in PTP activity among RAW and RAW Nramp-1 cells was maintained in response to a strong PTP-inducing stimulus and whether this difference plays an active role in the control of \textit{L. donovani} infection.

Infection of RAW M$$\phi$$s promotes a 35% induction of PTP activity above basal level. However, functional NRAMP-1
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expression limits the capacity of Mφs to reach full PTP activation in response to Ld (Fig. 4A), suggesting that the classical down-regulation of host cell signaling may be partially blocked in NRAMP-1-expressing cells. Knowing that parasite-induced Mφ PTP activation is fundamental for its intracellular development, we studied the capacity of Ld to survive in both cell lines by infecting Mφs with luciferase-transfected Ld promastigotes (Ld-Luc) (38). Following 6 h of initial Mφ-parasite contact and a chase period of 24 h, no difference in intracellular survival was observed between RAW and RAW Nramp-1 Mφs. However, when Mφs were activated with LPS following the initial 6-h Mφ-parasite contact, the intracellular parasite killing capacity of RAW Nramp-1 Mφs was greatly increased as a result of higher NO production. In contrast, RAW cells showed a limited capacity to fully control the infection, in line with low NO levels (Fig. 4, B and C). When RAW and RAW Nramp-1 Mφs were pretreated with bpV(phen) prior to infection and LPS stimulation, the intracellular Ld survival rate was greatly reduced in both cell lines as a result of general inhibition of PTP activity and downstream induction of NO (Fig. 4, B and C). However, a significant difference in the killing capacity of both cell lines was still evident, suggesting that although differential PTP activation plays an important role in the intracellular parasite killing, other PTP-independent events act together to fully control the infection.

Insights into the Mechanism of NRAMP-1-dependent PTP Activity Regulation—In an effort to understand the mechanism underlying the differential PTP activity associated with NRAMP-1, we sought to determine whether PTP protein expression was altered. Western blot analysis of a broad panel of PTPs, roughly accounting for an estimated 20% of leukocyte PTPs (55), showed no difference among RAW and RAW Nramp-1 cells in the expression levels of SHP-1, SHP-2, PTEN, TCPTP, PTP-PEST, PTP1B (Fig. 5A), MKP2, CD45, and KAP (data not shown). However, the specific activity of immunoprecipitated PTP1B and SHP-1, was 50 and 25% lower, respectively, in RAW Nramp-1 Mφs (Fig. 5B).

Similar PTP expression profiles in RAW and RAW Nramp-1 Mφs suggest that a post-translational regulatory mechanism may be responsible for the differential PTP activity. To address this, an in-gel PTP activity assay was performed. In this assay, PTPs are fully denatured and subsequently renatured, thus recovering their enzymatic activity. A post-translational modification affecting PTP activity, such as reversible inhibition by catalytic cysteine oxidation, protein–protein interactions, or protein phosphorylation, will be abrogated during the steps of in-gel protein denaturation and renaturation, and in-gel PTP activity will be restored (41, 42). As seen in Fig. 5C, a reversible mechanism of PTP inhibition in RAW Nramp-1 is favored, since a similar intensity of PTP bands ranging from 37 to 160 kDa is seen when comparing RAW and RAW Nramp-1 protein extracts. In contrast, a positive control of irreversible PTP inhibition is shown in lanes 3 and 4, where incubation of Mφs with the irreversible PTP inhibitor bpV(phen) prior to gel running results in the reduction of intensity and near disappearance of PTP bands (75–160 kDa).

The importance of divalent cations, such as calcium, zinc, and manganese, in the control of enzymatic reactions and intracellular signaling is well known. We therefore sought to determine whether metal substrates of NRAMP-1 may act as regulators of PTP activity. Total RAW Nramp-1 Mφ protein lysates were extracted and incubated with ferric citrate, ZnCl2, and MnCl2, or with chelators for iron (DFO) or zinc (TPEN), after which PTP activity was determined by pNPP hydrolysis. Interestingly, iron treatment inhibited PTP activity to the level of the PTP inhibitor bpV(phen), whereas zinc showed a modest 20% inhibition, and manganese showed no significant effect (Fig. 6A). Of interest, the specific activity of SHP-1 and PTP1B, two PTPs intimately involved in IFN-γ and LPS signaling, was significantly inhibited by iron treat-
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Inhibition in both cell lines was reached at a dose of 500 \( \mu M \), resulting from iron excess (Fig. 6C).

Cellular iron homeostasis is a tightly controlled process that involves the coordinated activity of extracellular iron import, intracellular storage, mobilization, utilization, and export (56). To mimic the effect of NRAMP-1 expression on PTP activity, we incubated RAW M\( \phi s \) ON with doses of ferric citrate ranging from 100 to 1000 \( \mu M \). Despite the use of high doses of extracellular iron, only partial PTP inhibition was observed at a subcytotoxic dose of 500 \( \mu M \), where PTP activity of RAW cells was inhibited to the level of basal activity in RAW Nramp-1 M\( \phi s \) (Fig. 6D). Incubation of RAW cells with ferric citrate prior to Ld-Luc infection and LPS stimulation promoted intracellular parasite killing similar to LPS-stimulated RAW Nramp-1 cells as determined by luciferase activity (Fig. 6E). These data clearly support an effect of iron, a metal substrate of NRAMP-1, in the regulation of PTP activity and downstream in the control of Leishmania infection.

**DISCUSSION**

NRAMP-1 has been associated with host resistance to intracellular pathogens, up-regulation of proinflammatory functions in M\( \phi s \), and more recently with susceptibility to autoimmune diseases (57, 58). Although major understanding has accumulated regarding the biochemistry and effector functions linked to NRAMP-1 (59), it is still unclear how as a divalent cation transporter it can mediate the pleiotropic effects associated with its functional expression. Lafuse and co-workers (30, 60) have proposed that NRAMP-1 expression influences the mRNA stability of several NRAMP-1-regulated genes, including major histocompatibility complex II, tumor necrosis factor-\( \alpha \), iNOS, and NRAMP-1 itself, via a mechanism involving a ROS-dependent signaling pathway requiring protein kinase C and mitogen-activated protein kinases (51). However, it remained elusive how NRAMP-1 metal transport influences protein kinases activities and ROS generation in order to promote the stabilization of certain mRNA species. In the present study, we propose a mechanism whereby NRAMP-1-mediated iron transport contributes to the regulation of M\( \phi \) functions through the modulation of PTP activity, linking divalent cation transport, signaling, and cellular functions.

Protein phosphorylation, as a result of the balanced action of protein kinases and protein phosphatases, is fundamental for the activation, localization, and substrate specificity of signaling molecules. In extension of previous observations (14, 50), we show that the higher nuclear translocation and DNA binding activity of NF-\( \kappa B \) and STAT1 in response to LPS and IFN-\( \gamma \) in NRAMP-1-expressing cells correlates with higher phosphorylation of protein members of the mitogen-activated protein kinase and JAK/STAT signaling pathways. Several PTPs have been shown to regulate LPS and IFN-\( \gamma \) signaling, including SHP-1 (32–34, 39, 61), PTP1B (62, 63), TCPTP (64, 65), mitogen-activated protein kinase phosphatases (66), and SHP-2 (67). In this line of thought, we sought to evaluate the status of PTP activity in response to NRAMP-1 expression. RAW Nramp-1 M\( \phi s \) had 35% lower PTP activity compared with NRAMP-1-deficient RAW cells. Of interest, the specific activity of two PTPs involved in the JAK/STAT and MEK/ERK sig-
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SIGNaling pathways, SHP-1 and PTP1B, was significantly lower in RAW Nramp-1 cells, which may contribute to the higher phosphorylation of MEK1/2, ERK1/2, and STAT1 and therefore to the enhanced NO production. Together with SHP-1 and PTP1B, TCPTP is involved in the regulation of JAK/STAT signaling (64, 65, 68, 69). Although protein expression of TCPTP was observed in both RAW and RAW Nramp-1 (Fig. 5A), the enzymatic activity of the immunoprecipitated protein was undetectable by biochemical substrate dephosphorylation assays (data not shown). This suggests that in our M/H9278 system, SHP-1 and PTP1B may exert a stronger regulatory function in the NRAMP-1-dependent modulation of IFN-γ signaling.

Natural resistance to Leishmania infection, as with other intracellular parasites, is associated with the functional expression of NRAMP-1. In addition, disease progression is exacerbated by a strong parasite-induced Mφ PTP activation, which leads to the negative regulation host cell functions (70). Several pathogens are capable of interfering with host cell signaling by modifying tyrosine phosphorylation, as is the case of Yersinia (71, 72), Salmonella (73), and Trypanosoma cruzi (74). However, only two cases have been documented where the pathogen itself (39, 75) induces host PTP activity as a mechanism to down-regulate host cell functions. We have used L. donovani infection to evaluate the contribution of NRAMP-1 in the regulation of Mφ PTP activation. As shown here, NRAMP-1 expression prevents full induction of PTP activity in infected Mφs, promoting positive signal transduction, which leads to NO production and greater leishmanicidal capacity upon LPS stimulation. The importance of NRAMP-1-dependent regulation of PTP activity in the outcome of Ld infection is moreover supported, given that NRAMP-1-deficient cells rescued the capacity to produce NO and control the intracellular parasite after their PTP activity was partially inhibited following treatment with the PTP inhibitor bpV(phen). This suggests that NRAMP-1 regulation of PTP activity is maintained even in a scenario where PTPs are strongly induced, as is the case of Ld infection, limiting the negative regulation of host cell functions induced by the parasite and promoting the control of infection.

Changes in cellular PTP activity may result from reversible or irreversible events, including differential protein expression, phosphorylation, oxidation, and dimerization (76, 77). Western blot analysis of a broad panel of M/H9278 PTPs suggests that NRAMP-1 regulation of PTP activity does not result from differential PTP expression; however, we do not discard the possibility that other PTPs not evaluated in this study may be differentially expressed. The dynamic nature of signaling cascades requires a tight regulation of PTP activity. An irreversible inhibition of PTPs will be detrimental for cellular stability, since no negative control of signal transduction will occur. Therefore, a reversible mechanism of PTP regulation by NRAMP-1 expression is fundamental for the proper display of Mφ functions. Our data suggest that the reduced PTP activity of RAW Nramp-1 Mφs indeed results from a reversible inhibition. Following PTP denaturation and renaturation in an in-gel PTP activity assay, active PTP bands in RAW Nramp-1 show similar intensities to NRAMP-1-deficient cells; this is explained by the fact that any reversible PTP inhibition, including oxidation or phosphorylation, will be abrogated after protein denaturation.
The PTP signature motif (I/V)HCXXGXXR(S/T) localized to the catalytic domain of the enzymes, is characterized by the presence of a conserved cysteine residue acting in the nucleophilic attack of the incoming phosphate group (78). The reduced state of this residue is fundamental for PTP activity and, when oxidized, impairs enzymatic activity (77). It has been recently demonstrated that ROS play a pivotal role in the reversible inhibition and regulation of PTPs (53, 77, 79, 80). Given that NRAMP-1 expression has been associated with increased intracellular ROS (16, 81), this event may contribute to the decreased PTP activity seen in our experimental system. In addition to ROS, recent reports show that zinc inhibits PTP activity in vitro and in vivo (82–84). Following this line of thought, we have evaluated the impact of NRAMP-1 expression on Mφ PTP activity. As shown in Fig. 6A, zinc inhibits PTP activity by 20%; however, iron, a well studied metal substrate of NRAMP-1, inhibited total PTP activity up to the levels of the specific PTP inhibitor bpV(phen). This interesting finding led us to further investigate the role of iron in PTP activity regulation. In support of a mechanism where NRAMP-1 regulation of PTP activity is mediated by its iron transport function, stronger PTP inhibition was observed in RAW Nramp-1 Mφs at lower doses of ferric citrate compared with NRAMP-1-deficient cells (Fig. 6C). In intact cells, iron pretreatment of RAW Mφs mimicked the effect of NRAMP-1 expression by partially inhibiting PTP activity and promoting Leishmania killing, in accordance with previous reports where iron overload in BALB/c mice prevented the onset and development of lesion formation following L. major infection (85, 86). Moreover, the finding that iron treatment by itself inhibits the activity of both PTP1B and SHP-1 suggests that differential iron transport may play a role in the regulation of LPS and IFN-γ signaling and consequently in NO production by directly affecting PTP activity. These data give clear evidence for the involvement of iron in the NRAMP-1 regulation of signaling pathways and control of Leishmania infection.

In conclusion, our findings suggest a model that directly links NRAMP-1 metal transport and the regulation of Mφ functions. NRAMP-1-mediated transport of zinc and iron from the late endosome/lysosome and phagolysosomal compartment of Mφs directly or indirectly (by catalyzing the formation of ROS) inhibit PTP activity in a reversible fashion, promoting protein phosphorylation and positive signal transduction, leading to the up-regulation of effector Mφ functions, in our particular case of study nitric oxide production. In addition to NRAMP-1-mediated regulation of protein kinase activities and mRNA stability, our current study on the regulation of PTP activity broadens our understanding of the complex mechanism responsible for the NRAMP-1-mediated modulation of cellular functions, bringing together basic metal transport and signal transduction.

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