Slc11a1-mediated resistance to *Salmonella enterica* serovar Typhimurium and *Leishmania donovani* infections does not require functional inducible nitric oxide synthase or phagocyte oxidase activity

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Abstract: Solute carrier family 11a member 1 (*Slc11a1*; formerly natural resistance-associated macrophage protein 1) encodes a late endosomal/lysosomal protein/divalent cation transporter, which regulates iron homeostasis in macrophages. During macrophage activation, Slc11a1 exerts pleiotropic effects on gene regulation and function, including generation of nitric oxide (NO) via inducible NO synthase (iNOS; encoded by *Nos2A*) and of reactive oxygen intermediates (ROI) via the phagocyte oxidase complex. As NO and ROI have potent antimicrobial activity in macrophages, it was assumed that their activities would contribute to *Slc11a1*-regulated innate resistance to *Salmonella enterica* serovar Typhimurium and *Leishmania donovani*. By intercrossing mice with gene disruptions at *Nos2A* and *Cybb* (encoding gp91phox, the heavy chain subunit of cytochrome b-245 and an essential component of phagocyte NADPH oxidase) onto equivalent *Slc11a1* wild-type and mutant genetic backgrounds, we demonstrate that neither iNOS nor gp91phox activity is required for *Slc11a1*-mediated innate resistance to either infection. Functional gp91phox and iNOS are required to control *S. enterica* serovar Typhimurium in non-*Slc11a1*-regulated phases of infection. For *L. donovani*, an organ-specific requirement for iNOS to clear parasites from the spleen was observed at 50 days post-infection, but neither iNOS nor gp91phox influenced late-phase infection in the liver. This contrasted with *Leishmania major* infection, which caused rapid lesion growth and death in iNOS knockout mice and some exacerbation of disease with gp91phox deficiency. This highlights the adaptive differences in tissue and cellular tropisms between *L. donovani* and *L. major* and the different genes and mechanisms that regulate visceral versus cutaneous forms of the disease. *J. Leukoc. Biol.* 77: 311–320; 2005.

Key Words: *Nramp1 · divalent cation transporter · macrophage activation*

INTRODUCTION

Solute carrier family 11a member 1 [*Slc11a1*; formerly natural resistance-associated macrophage protein 1 (*Nramp1*)] encodes a late endosomal/lysosomal divalent cation transporter, which regulates iron homeostasis in macrophages (reviewed in refs. [1–4]). During macrophage activation, Slc11a1 exerts pleiotropic effects, including quantitative differences in induction of early response genes such as the CXC chemokine KC (CXCL1 or murine Gro-α) [5], interleukin-1β [6], and tumor necrosis factor α [7] at protein and/or mRNA levels, as well as iron- [8] and interferon-γ/lipopolysaccharide [8, 9]-regulated differences in expression and stability of mRNA for inducible nitric oxide synthase (iNOS) encoded by *Nos2A*. Differences in NADPH oxidase activity have also been observed in response to phorbol myristate acetate in RAW264.7 macrophages, stably transfected with constructs carrying wild-type or mutant (mt) *Slc11a1* [10]. In vitro, studies of iNOS induction and use of inhibitors have shown that NO generated by iNOS activity is responsible for macrophage antimicrobial activity against numerous intracellular pathogens (reviewed in refs. [11–13]), including *Salmonella enterica* serovar Typhimurium [14], *Mycobacterium bovis* [15], and *Leishmania donovani* [16], which come under innate *Slc11a1* control in the early phases of infection. It was assumed, therefore, that iNOS activity might contribute to early *Slc11a1*-mediated resistance to infection. Similarly, reactive oxygen intermediates (ROI), generated by NADPH oxidase, have antimicrobial activity against all three groups of the pathogen in vitro [17–19], and it had been proposed [20] that the interaction of ferrous ions (Fe^{2+}) trans-

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ported by Slc11a1 with ROI via the Fenton and Haber-Weiss reactions might contribute to the generation of more toxic radicals in Slc11a1 wild-type but not mt macrophages. By intercrossing mice with gene disruptions at *Nos2A* and *Cybb* (encoding gp91phox, the heavy chain subunit of cytochrome b-245 and an essential component of phagocyte NADPH oxidase) onto equivalent *Slc11a1* wild-type and mt genetic backgrounds, we demonstrate that neither iNOS nor gp91phox activity is required for *Slc11a1*-mediated innate resistance to *S. enterica* serovar Typhimurium or *L. donovani* infection. We show that functional gp91phox and iNOS activities are required for non-*Slc11a1*-regulated early and late phase of *S. enterica* serovar Typhimurium infection, respectively. However, only an organ-specific requirement for iNOS was observed in clearance of parasites from spleen but not liver in the later, non-*Slc11a1*-regulated phases of *L. donovani* infection. This contrasts with rapid lesion growth and death in *Nos2A*$^{-/-}$ knockout mice following *Leishmania major* infection, with some exacerbation of disease also observed with *Cybb*$^{-/-}$ gene disruption. This highlights the adaptive differences in tissue and cellular tropisms between *L. donovani* and *L. major* and the different genes and mechanisms that regulate visceral versus cutaneous forms of the disease.

MATERIALS AND METHODS

Mice

Congenic N20 B10.L-*Lsh* (=*Nramp1*; =*Slc11a1*) were bred in-house [21] following 20 generations of backcrossing the *Slc11a1* wild-type allele from C57L mice onto a C57BL/10ScSn (=B10) background. These are congenic with *Slc11a1* mt B10 mice except for the ~10 Mb interval distal to D1Mit80 and proximal to D1Mit44 on chromosome 1, which carries the *Slc11a1* gene. To produce new lines that were homozygous *Slc11a1* wild-type (=*Slc11a1*$^{+/+}$) but carried gene disruptions at *Nos2A* (=*Nos2A*$^{-/-}$) or *Cybb* (=*Cybb*$^{-/-}$), founder *Nos2A*$^{-/-}$ [22] and *Cybb*$^{-/-}$ [23] were intercrossed with N20 mice. The *Nos2A*$^{-/-}$ produced by Laubach and co-workers [22] was reported to have undergone six generations of backcrossing onto a C57BL/6J (=B6) background prior to our acquiring it. As B6 mice are *Slc11a1* mt, we expected to obtain double heterozygous mice for *Slc11a1*$^{+/mt}$ and *Nos2A*$^{+/-}$ at the F1 of the first intercross. In the event, all mice were homozygous *Slc11a1*$^{+/+}$, indicating that the region of chromosome 1 carrying *Slc11a1* in the original line [19] was still homozygous for the 129 background in which the *Nos2A* knockout was generated. These F1 mice were further intercrossed, and *Nos2A*$^{+/+}$ and *Nos2A*$^{-/-}$ mice were selected to establish breeding colonies (by brother-sister matings of *Nos2A*$^{+/+}$×*Nos2A*$^{+/+}$ or *Nos2A*$^{-/-}$ ×*Nos2A*$^{-/-}$ F2 progeny from the same F1 intercross) of double homozygous *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ and *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ on the intercrossed B6/B10 background at F3. The founder *Cybb*$^{-/-}$ mice were also on a B6 background, and in this case, the expected double heterozygous mice for *Slc11a1*$^{+/mt}$ and *Cybb*$^{+/-}$ were obtained at the F1 of the first intercross. *Slc11a1* and *Cybb* genotyping was performed using tail DNA from all mice and selective breeding used to establish double homozygous *Slc11a1*$^{+/+}$.*Cybb*$^{+/+}$ and *Slc11a1*$^{+/+}$.*Cybb*$^{-/-}$ lines at F3 on the intercrossed B6/B10 background. For both sets of mice, infections were compared with *Slc11a1*$^{mt/mt}$, *Nos2A*$^{+/+}$, and *Cybb*$^{+/+}$ B10 or B6 mice as indicated. BALB/c mice (Harlen Olac, Bicester, UK) were used as susceptible controls for *L. major* infection experiments. All breeding and procedures were carried out under license and UK Government Home Office regulations. Mice were between 6 and 16 weeks old over all infection experiments but age-matched ±2 weeks and sex-matched within each experiment as indicated below.

Genotyping

During the breeding protocols, the *Nos2A*$^{-/-}$ gene-disrupted allele for *Nos2A* was genotyped on *Bam*HI-digested, genomic DNA Southern blots hybridized with a *Nos2A* intron 10 probe amplified by polymerase chain reaction (PCR) from genomic DNA using forward (5′-CTGGACCAGGCCACTTTG-3′) and reverse (5′-CCTCCACTTGTCCATCCATT-3′) primers. Diagnostic bands of 14 kb and 5 kb distinguished *Nos2A* wild-type from gene-disrupted alleles, respectively. Similarly, the *Cybb*$^{-/-}$ gene-disrupted allele for *Cybb* was genotyped on *Eco*RI-digested, genomic DNA Southern blots, hybridized with a *Cybb* exon 2 probe, amplified by PCR from genomic DNA using forward (5′-CAAGATTGCATGAGGCAGAA-3′) and reverse (5′-CAACTTTTCCTGTTT-GTGCAG-3′) primers. Diagnostic bands of 13 kb and 6.8 kb distinguished *Cybb* wild-type from gene-disrupted alleles, respectively. *Slc11a1* genotyping was undertaken by sequence analysis across the mutation site following amplification of a 492-bp product from genomic DNA using exon 5 forward (5′-ATATGCAGGAAGTCATCGGG-3′) and intron 6 reverse (5′-CAAGAT-GAGGGGCTTTCTCTC-3′) primers, with the forward primer used as the sequencing primer.

Salmonella infection and monitoring

For intravenous (i.v.) inoculation into mice, virulent *S. enterica* serovar Typhimurium C5 was grown at 37°C as stationary, overnight cultures in Luria-Bertani (LB) broth (Difco, Becton Dickinson, Cowley, Oxford, UK). Aliquots were snap-frozen and stored in liquid nitrogen. The inoculum was diluted in phosphate-buffered saline (PBS) and injected into the lateral tail vein. The number of viable bacteria in each inoculum was checked by dilution and pour-plating onto LB agar plates. To enumerate bacterial load in the organs, spleens and livers of mice (four per time-point per strain) were removed aseptically and homogenized in a Colworth Stomacher in 10 ml cold, distilled water [24]. Viable counts were determined using pour plates of LB agar. For 50% lethal dose (LD_{50}) determinations, groups of mice (four to six per dose per strain) were injected i.v. with 10- or 20-fold decreasing doses of *S. enterica* serovar Typhimurium C5 over the range 10^5–10^1, and mortality scored over a 30-day period. LD_{50} values were calculated according to the method of Reed and Muench [25]. Age-matched (±2 weeks) male mice were used for *S. enterica* serovar Typhimurium experiments as indicated.

Leishmania infection and monitoring

To determine response to cutaneous leishmaniasis, mice (five to six per strain) were inoculated subcutaneously (s.c.) into the right-hind footpad with 2×10^6 stationary-phase, metacyclic promastigotes of *L. major* strain LV39, cultured in Schneider's insect medium (Sigma, Poole, UK), supplemented with 10% fetal calf serum (Invitrogen, Paisley, UK) and 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen). Infection was monitored as increase in footpad depth, measured using digital Vernier callipers. To conform to UK Home Office regulations, euthanasia of mice was carried out when footpad lesions became ulcerated and necrotic. Experience shows that this level of pathology is always lethal. To determine response to visceral leishmaniasis, mice (three to four per time-point per strain) were inoculated i.v. into the lateral tail vein with 2×10^7 or 5×10^7 amastigotes freshly harvested from spleens of hamsters infected with *L. donovani* strain LV9 as described [26]. Groups of mice were killed on days 1, 4, 7, and 15 and ~50 days post-infection as indicated. Parasite loads were enumerated microscopically from KWIK™DIFF (Thermo Shandon, Pittsburgh, PA)-stained impression smears of spleen and liver. Results are expressed as Leishman Donovan units (LDU) [26], which equate to the number of parasites per 1000 organ cell nuclei multiplied by the organ weight in mg. Age-matched (±2 weeks), female mice were used for *Leishmania* experiments as indicated.

Immunohistology

Liver segments from N20 mice harvested 2–4 days post *L. donovani* infection were mounted in optimal cutting temperature compound (CellPath, Powys, UK) and snap-frozen. For low-power fluorescence microscopy, cryostat sections (8 μm thick) were warmed to room temperature, fixed for 10 min in 4% w/v paraformaldehyde (BDH, Poole, UK) in PBS (pH 7.4), and washed 2× in PBS for 5 min. Sections were post-fixed by immersion in 2:1 ethanol:acetic acid at 4°C and washed 2× in PBS for 5 min. Nonspecific staining was reduced by incubation for 1 h with 3% fatty acid-free bovine serum albumin in Triton–Tris-buffered saline (154 mM NaCl, 10 mM Tris, 0.01% sodium azide, 0.1% Triton X-100, pH 7.4) prior to incubation with pooled, primary monoclonal antibody (Ab) rat anti-mouse M1/70 Ab (Harlan Sera-Lab, Loughborough, UK, 1:10) and affinity-purified polyclonal rabbit anti-iNOS Ab (Affiniti Research Products, Exeter, UK, 1:500). M1/70

recognizes CD11b, the β-chain of the type-3 complement receptor [27]. The anti-iNOS Ab was raised against a 21-kDa protein fragment corresponding to amino acids 961–1144 of the mouse macrophage iNOS protein [28] and detects the 130-kDa full-length protein in macrophages from *Nos2A*$^{+/+}$ mice, which was completely absent in macrophages from *Nos2A*$^{-/-}$ mice (data not shown). After washing, slides were coincubated in fluorescein isothiocyanate (FITC)-conjugated, affinity-purified donkey anti-rat immunoglobulin G (IgG; Jackson Immuno Research Laboratories, West Grove, PA, 1:50) and affinitiy-purified tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Sigma, 1:20) for 5 h at ambient temperature. Sections were washed 3× in PBS, rinsed in distilled water, and mounted in Citifluor AFI (Agar Scientific, Kent, UK). Staining was viewed with an Olympus Provis AX70 fluorescent microscope (London, UK), mounted with a Hamamatsu C47-42-95 digital camera (Photonics KK, Japan), and images were captured with Openlab software. Exposures for FITC and TRITC channels varied between 75 and 400 ms. The microscope, camera, and printer settings were standardized within experiments for stained and control sections, all of which had been incubated in parallel. For high-power confocal microscopy, 6 μm cryostat sections were incubated in primary Ab simultaneously. The first primary Ab was an affinity-purified polyclonal anti-Slc11a1 Ab (1:1000) raised against the N-terminal 82 amino acids of the Slc11a1 protein [29] and was a gift from Dr. C. Howard Barton (University of Southampton, UK). This Ab recognizes the mature, fully glycosylated, 90–100 kDA Slc11a1 band on Western blots of Slc11a1 wild-type macrophages [30]. The second primary Ab was M1/70 (1:800) or monoclonal rat anti-mouse F4/80 (Serotec, Oxford, UK, 1:50). Secondary Ab were Alexa^{TM}594-coupled goat anti-rat IgG (1:200) and Alexa^{TM}488-coupled goat anti-rabbit IgG (1:400, Molecular Probes, Leiden, Netherlands). Slides with cryostat sections were fixed for 10 min at 4°C with 100% acetone, air-dried, and then rinsed in PBS and blocked for 1 h at ambient temperature in 0.1% Tween-20/PBS supplemented with normal goat serum (1:40). Primary Ab, diluted in blocking buffer, were incubated for 1 h at ambient temperature. Slides were washed 3× for 5 min in 0.1% Tween-20/PBS. Secondary Ab, diluted in blocking buffer, were incubated for 1 h at ambient temperature. Slides were washed as above, rinsed in PBS, and then mounted in Prolong Antifade (Molecular Probes) and viewed with a Nikon optiphot-2 epifluorescence microscope coupled to a Bio-Rad MRC 1000 confocal laser-scanning attachment (Bio-Rad Laboratories Ltd., Hemel Hempsted, UK). Images were collected using Lasersharp 2000 software.

Statistics

All footpad measurements and parasite loads were determined blind to the experimental group. Unpaired, two-tailed Student's *t*-tests were used to compare bacterial or parasite loads between mouse strains. Differences between experimental groups were considered significant for P values <0.05.

RESULTS

Nos2A$^{-/-}$ does not compromise early *Slc11a1*$^{+/+}$ regulation of *S. enterica* serovar Typhimurium infection

Previous studies show that *Slc11a1* mediates its influence on virulent *S. enterica* serovar Typhimurium C5 during the acute phase (<7 days) of infection [31, 32]. LD_{50} values, 1 month after infection with tenfold decreasing doses (10^5, 10^4, 10^3, 10^2) of *S. enterica* serovar Typhimurium C5, indicated that F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ mice were intermediate in susceptibility compared with B10 *Slc11a1*$^{mt/mt}$.*Nos2A*$^{+/+}$ and F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ mice (data not shown). However, survival plots (**Fig. 1A**) following infection of F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ and *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ mice with 10^3 *S. enterica* serovar Typhimurium C5 showed that the *Nos2A*gene disruption does not influence Slc11a1-mediated resistance (100% survival) over the first 9 days of infection in F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ mice. In contrast, 100% mortality was observed in *Slc11a1*$^{mt/mt}$ B10 mice by day 8 post-infection. Failure of the *Nos2A* gene disruption to compromise early *Slc11a1*-mediated resistance to infection is further supported by monitoring liver (Fig. 1B) and spleen (Fig.

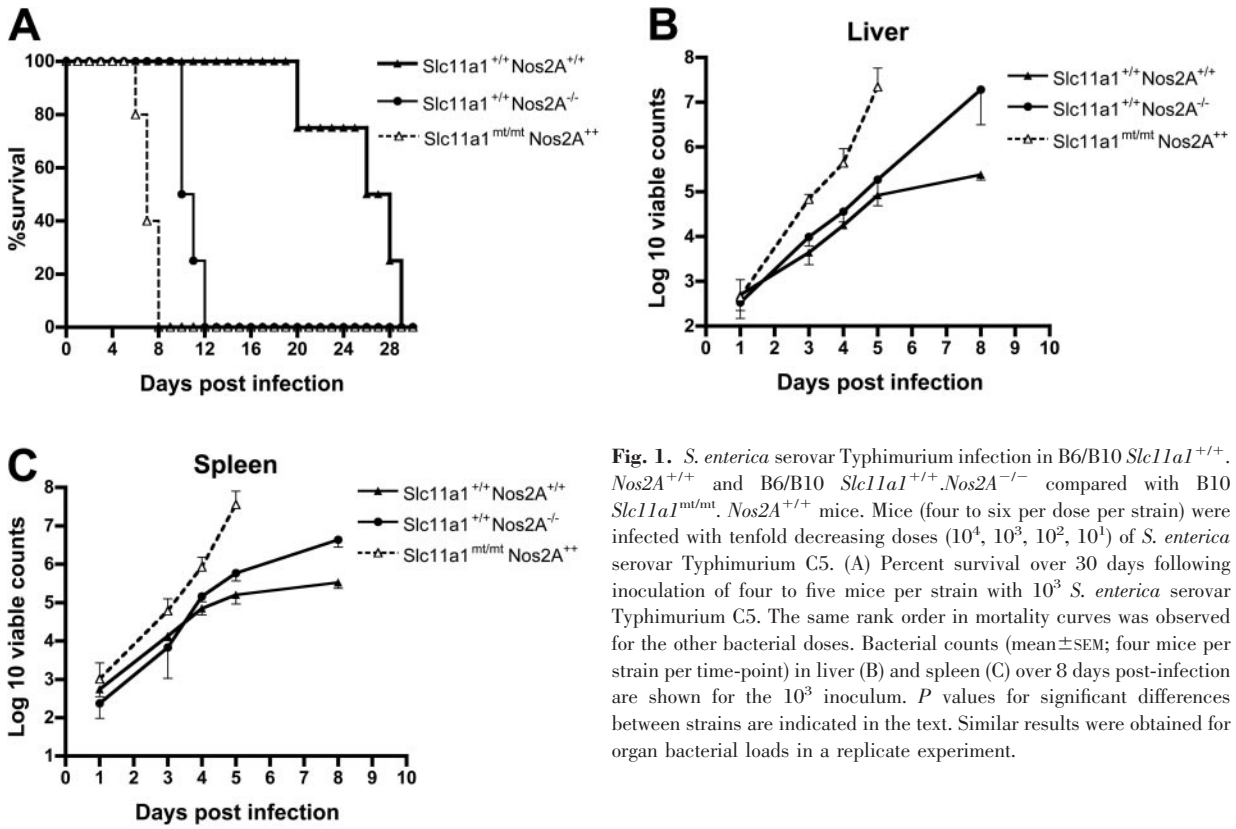

Fig. 1. *S. enterica* serovar Typhimurium infection in B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ and B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ compared with B10 *Slc11a1*$^{mt/mt}$.*Nos2A*$^{+/+}$ mice. Mice (four to six per dose per strain) were infected with tenfold decreasing doses (10^4, 10^3, 10^2, 10^1) of *S. enterica* serovar Typhimurium C5. (A) Percent survival over 30 days following inoculation of four to five mice per strain with 10^3 *S. enterica* serovar Typhimurium C5. The same rank order in mortality curves was observed for the other bacterial doses. Bacterial counts (mean±SEM; four mice per strain per time-point) in liver (B) and spleen (C) over 8 days post-infection are shown for the 10^3 inoculum. P values for significant differences between strains are indicated in the text. Similar results were obtained for organ bacterial loads in a replicate experiment.

1C) bacterial counts over time following infection with 10^3 *S. enterica* serovar Typhimurium C5. In this case, all *Slc11a1*$^{mt/mt}$ B10 mice were dead by day 7 post-infection, and no significant differences in liver or spleen bacterial loads were observed between F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$, *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$, or B10 background N20 *Slc11a1*$^{+/+}$ (data not shown) mice over days 1–5 post-infection. *Nos2A*$^{-/-}$ gene disruption did influence post-*Slc11a1* phase, as evidenced by significant differences in spleen ($P<0.008$) bacterial counts for *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ mice compared with *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ mice at day 8 post-infection, all *Nos2A* gene-disrupted mice were dead by day 9, and *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ mice all survived beyond this time-point in this experiment (data not shown).

Nos2A$^{-/-}$ does not compromise early *Slc11a1*$^{+/+}$ regulation of *L. donovani* infection

In contrast to *S. enterica* serovar Typhimurium infection, *L. donovani* does not cause acute mortality in mice. Expansion of the parasite population in liver and spleen is slower, and *Slc11a1* resistance can be measured over the first 7–15 days of infection [33]. Confirmation that *Nos2A* plays no role in *Slc11a1*-mediated resistance to infection in spleen is demonstrated in **Figure 2** by failure to observe significant differences in parasite loads in F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$, *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$, or B10 background N20 *Slc11a1*$^{+/+}$ mice (data not shown) at days 4, 7, or 15 post-infection. Although there was a trend toward higher parasite load in F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ compared with F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ in the liver at days 7 and 15 post-infection, these did not achieve statistical significance. In contrast, significantly higher parasite loads were observed by days 7 ($P=0.002$, 0.004, 0.016) and 15 ($P=0.004$, 0.004, 0.005) post-infection in the liver and by day 15 ($P=0.0005$, 0.0002, 0.0001) in the spleen, when B10 *Slc11a1*$^{mt/mt}$.*Nos2A*$^{+/+}$ mice were compared with the three *Slc11a1*$^{+/+}$ mouse lines F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$, *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$, or B10 background N20 *Slc11a1*$^{+/+}$, respectively. It is interesting that gene disruption at *Nos2A*$^{-/-}$ did compromise the ability of F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ to clear parasites in the spleen ($P=0.004$) but not the liver at day 50 compared with F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ mice (Fig. 2), a period during which T cell-mediated immunity under genetic regulation of H-2 is known to overcome early failure to control parasite expansion in liver and spleen in B10 *Slc11a1*$^{mt/mt}$ mice [31]. It should be noted, however, that parasite loads were low in the spleens of all strains at this time-point, so the difference caused by the *Nos2A*$^{-/-}$ was small. We did not intercross the *Nos2A*$^{-/-}$ into the B10 *Slc11a1*$^{mt/mt}$ line, where the effect may have been more pronounced.

Nos2A$^{-/-}$ does compromise resistance to *L. major* infection

Previous studies have shown that *Slc11a1* does not regulate cutaneous lesion expansion caused by s.c. inoculation of *L. major* parasite [34] or expansion of the parasite population

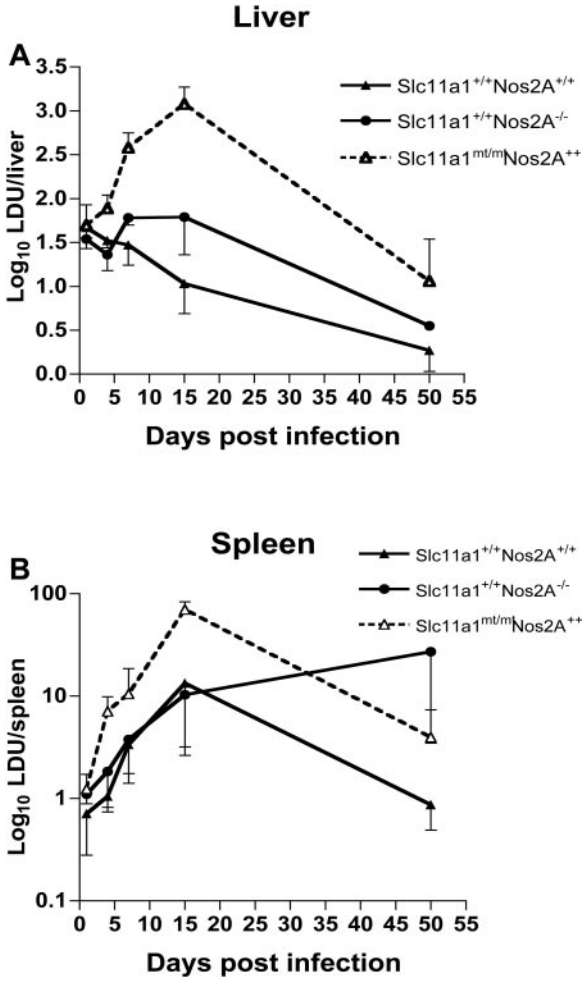

Fig. 2. *L. donovani* infection in B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ and B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ compared with B10 *Slc11a1*$^{mt/mt}$.*Nos2A*$^{+/+}$ mice. Parasite loads (LDU; mean±SEM; three or four mice per strain per time-point) were measured in liver (A) and spleen (B) at intervals over 50 days post-infection with 5×10^7 *L. donovani* LV9 amastigotes. *P* values for significant differences between strains are indicated in the text. Similar results were obtained for organ parasite loads in a replicate experiment.

in liver or spleen following i.v. infection [35]. In contrast, *Nos2A* is crucial to regulation of the parasite in the skin and spread from lesions to the viscera [36, 37]. Consistent with these observations, our F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ mice were highly susceptible to s.c. infection with *L. major* (**Fig. 3**) compared with F3 B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ ($P<0.001$, days 14 and 21) or B10 background N20 *Slc11a1*$^{+/+}$ (Fig. 3 inset; $P<0.001$, days 14 and 21) mice. Indeed, lesion expansion in these mice exceeded (100% euthanasia by day 21) that observed in BALB/c (100% euthanasia by day 28) mice, classically recognized as the most susceptible mouse strain in which susceptibility to *L. major* infection comes under separate and complex genetic regulation. The results confirmed the previous observations [34, 35] that *Slc11a1* does not influence *L. major* infection, as B10 *Slc11a1*$^{mt/mt}$ and congenic N20 *Slc11a1*$^{+/+}$ lines did not differ significantly in lesion size over >50 days of infection (Fig. 3 inset).

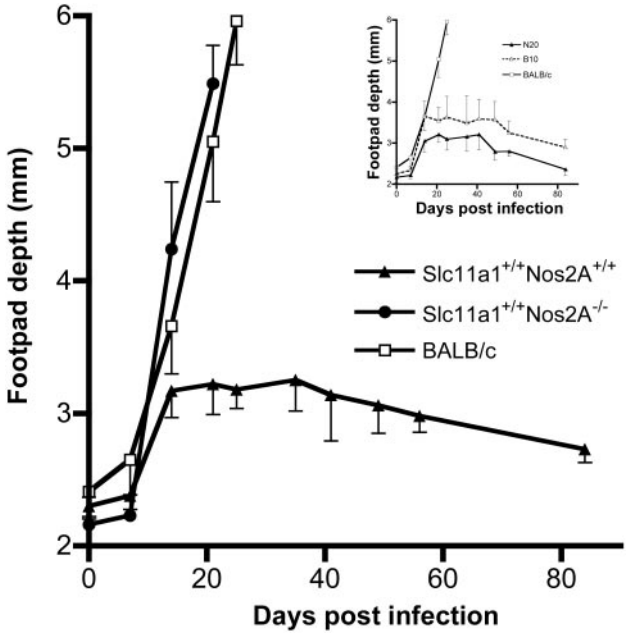

Fig. 3. *L. major* infection in B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$ and B6/B10 *Slc11a1*$^{+/+}$.*Nos2A*$^{-/-}$ compared with BALB/c mice. Infection was measured as footpad depth (mean±SEM; five or six mice per strain) at intervals over 86 days post-infection with 2×10^6 *L. major* LV39 metacyclic promastigotes. The inset shows parallel data for congenic B10 (*Slc11a1*$^{mt/mt}$.*Nos2A*$^{+/+}$) and N20 (*Slc11a1*$^{+/+}$.*Nos2A*$^{+/+}$) mice. *P* values for significant differences between strains are indicated in the text. This experiment replicated previous data [38] and was not repeated.

Cybb$^{-/-}$ does not influence *Slc11a1*$^{+/+}$ regulation of *S. enterica* serovar Typhimurium infection

In contrast to *Nos2A*$^{-/-}$, gene disruption at *Cybb* has a dramatic influence on early survival of mice following infection with 10^1 *S. enterica* serovar Typhimurium C5 (**Fig. 4A**). Nevertheless, *Slc11a1*$^{+/+}$.*Cybb*$^{-/-}$ survived longer than their *Slc11a1*$^{mt/mt}$.*Cybb*$^{-/-}$ counterparts, indicating that the *Slc11a1*-resistance mechanism was at least partially functional in the absence of functional gp91phox activity. This observation was borne out by following bacterial counts in liver (Fig. 4B) and spleen (Fig. 4C) over the first 6 days of infection, where *Slc11a1*$^{+/+}$.*Cybb*$^{-/-}$ showed reduced counts ($P<0.001$, day 2) compared with *Slc11a1*$^{mt/mt}$.*Cybb*$^{-/-}$ mice, comparable with the differences observed when *Slc11a1*$^{+/+}$.*Cybb*$^{+/+}$ mice were compared ($P<0.001$, days 2, 4, and 6) with *Slc11a1*$^{mt/mt}$.*Cybb*$^{+/+}$ mice. This is demonstrated most clearly by the inset graphs in Figure 4, B and C, which show bacterial counts in liver and spleen expressed as a percent of the day 1 counts within each strain. Here, is it clear that the two *Slc11a1*$^{+/+}$ strains (solid lines) show reduced rates of expansion of the bacterial population compared with the two *Slc11a1*$^{mt/mt}$ strains (dotted lines) on *Cybb*$^{+/+}$ and *Cybb*$^{-/-}$ backgrounds. These results indicate that gp91phox activity is essential for early control of bacterial expansion but by a mechanism separate from Slc11a1 regulation of the bacterial load in liver and spleen.

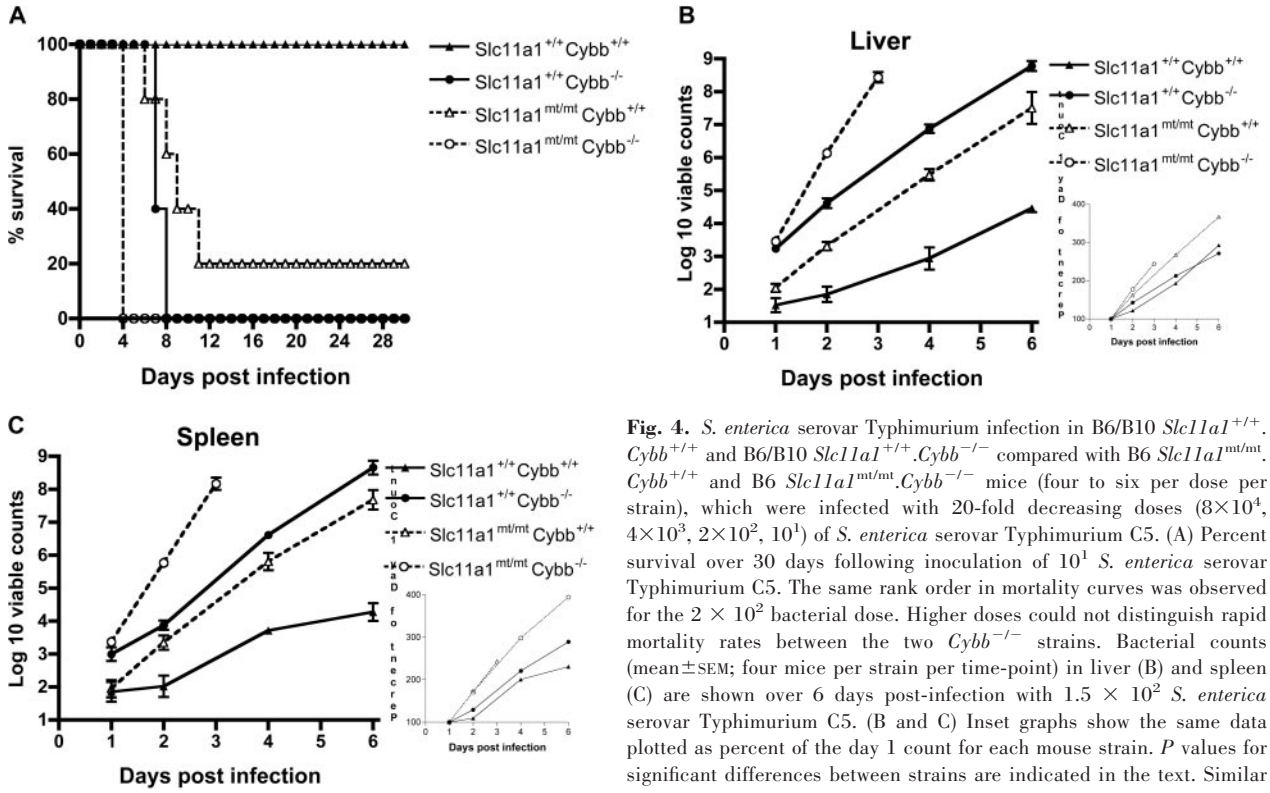

Fig. 4. *S. enterica* serovar Typhimurium infection in B6/B10 *Slc11a1*$^{+/+}$.*Cybb*$^{+/+}$ and B6/B10 *Slc11a1*$^{+/+}$.*Cybb*$^{-/-}$ compared with B6 *Slc11a1*$^{mt/mt}$.*Cybb*$^{+/+}$ and B6 *Slc11a1*$^{mt/mt}$.*Cybb*$^{-/-}$ mice (four to six per dose per strain), which were infected with 20-fold decreasing doses (8×10^4, 4×10^3, 2×10^2, 10^1) of *S. enterica* serovar Typhimurium C5. (A) Percent survival over 30 days following inoculation of 10^1 *S. enterica* serovar Typhimurium C5. The same rank order in mortality curves was observed for the 2×10^2 bacterial dose. Higher doses could not distinguish rapid mortality rates between the two *Cybb*$^{-/-}$ strains. Bacterial counts (mean±SEM; four mice per strain per time-point) in liver (B) and spleen (C) are shown over 6 days post-infection with 1.5×10^2 *S. enterica* serovar Typhimurium C5. (B and C) Inset graphs show the same data plotted as percent of the day 1 count for each mouse strain. *P* values for significant differences between strains are indicated in the text. Similar results were obtained for organ bacterial loads in a replicate experiment.

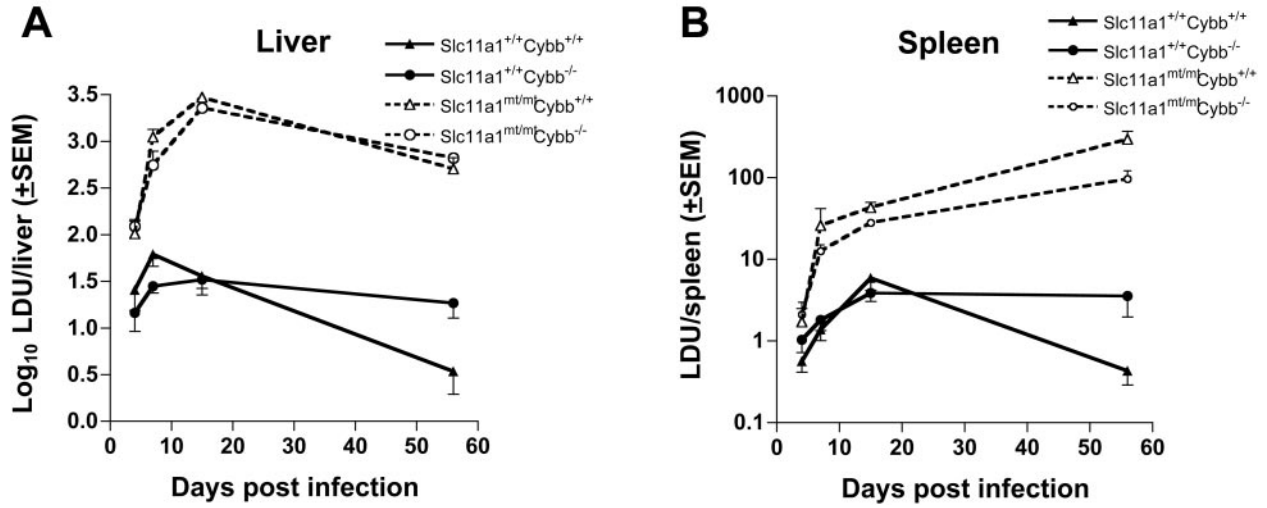

Fig. 5. *L. donovani* infection in B6/B10 *Slc11a1*$^{+/+}$.*Cybb*$^{+/+}$ and B6/B10 *Slc11a1*$^{+/+}$.*Cybb*$^{-/-}$ compared with B6 *Slc11a1*$^{mt/mt}$.*Cybb*$^{+/+}$ and B6 *Slc11a1*$^{mt/mt}$.*Cybb*$^{-/-}$ mice. Parasite loads (LDU; mean±SEM; four mice per strain per time-point) were measured in liver (A) and spleen (B) at intervals over 50 days post-infection with 2×10^7 *L. donovani* LV9 amastigotes. *P* values for significant differences between strains are indicated in the text. Similar results were obtained for organ parasite loads in a replicate experiment.

Cybb$^{-/-}$ does not compromise *Slc11a1*$^{+/+}$ regulation of *L. donovani* infection

As for *Nos2A*$^{-/-}$, gene disruption at *Cybb*$^{-/-}$ had no influence on the regulation of parasite loads in the livers and spleens over the initial *Slc11a1*-regulated phase of infection following i.v. inoculation of *L. donovani* or on the later, T cell-regulated, acquired immune response (**Fig. 5**). Differences observed at day 56 between F3 B6/B10 *Slc11a1*$^{+/+}$.*Cybb*$^{-/-}$ and *Slc11a1*$^{+/+}$.*Cybb*$^{+/+}$ in liver and spleen were not statistically significant nor was any effect of *Cybb*$^{-/-}$ seen on the B6 *Slc11a1*$^{mt/mt}$ background (Fig. 5). In contrast, differences between *Slc11a1*$^{+/+}$ mice and *Slc11a1*$^{mt/mt}$ mice were significant at $P < 0.001$ from day 7 onward, whether they were *Cybb*$^{+/+}$ or *Cybb*$^{-/-}$. B6 background *Slc11a1*$^{mt/mt}$ mice demonstrated a reduced ability to clear parasites from liver and spleen compared with B10 background *Slc11a1*$^{mt/mt}$ mice (data not shown), indicating that other genes on this background influence the H-2^b-regulated clearance of parasites observed in B10 mice [39]. However, this was not influenced by *Cybb*$^{-/-}$ gene disruption.

Cybb$^{-/-}$ does compromise resistance to *L. major* infection

Although gene disruption at *Nos2A*$^{-/-}$ had a dramatic and early influence on lesion growth and mortality following *L. major* infection on a mixed B6/B10 *Slc11a1*$^{+/+}$ background, gene disruption at *Cybb*$^{-/-}$ had only a small but significant influence on lesion size in B6 *Slc11a1*$^{mt/mt}$ (**Fig. 6A**;

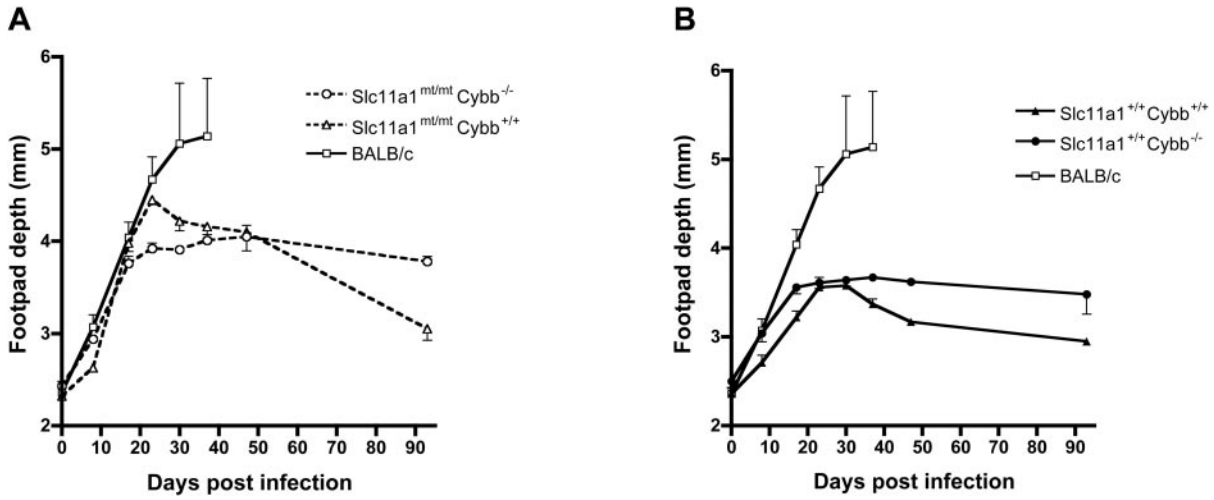

Fig. 6. *L. major* infection in (A) B6 *Slc11a1*$^{mt/mt}$.*Cybb*$^{+/+}$ and B6 *Slc11a1*$^{mt/mt}$.*Cybb*$^{-/-}$ mice or (B) B6/B10 *Slc11a1*$^{+/+}$.*Cybb*$^{+/+}$ and B6/B10 *Slc11a1*$^{+/+}$.*Cybb*$^{-/-}$ mice, compared in each case with BALB/c mice. Infection was measured as footpad depth (mean±SEM; five mice per strain) at intervals over 94 days post-infection with 2×10^6 *L. major* LV39 metacyclic promastigotes. *P* values for significant differences between strains are indicated in the text. This experiment was only performed once.

$P=0.0006$, day 93) and mixed B6/B10 *Slc11a1*$^{+/+}$ (Fig. 6B; $P=0.0017$, 3×10^6, and 0.031 at days 37, 47, and 93, respectively) mice in the later phases of the infection. However, there was no demonstrable pathology (ulceration) associated with this effect. Opposing effects of *Cybb*$^{-/-}$ were observed on B6 compared with mixed B6/B10 backgrounds at earlier timepoints (days 17 and 23), indicating variable influence of background genes, again with no demonstrable influence on pathology other than increased footpad swelling. As before, there were no significant differences between B10 *Slc11a1*$^{mt/mt}$ and N20 *Slc11a1*$^{+/+}$ mice in this experiment (data not shown), consistent with previous data (Fig. 3 inset and refs. [34, 35]), showing no influence of Slc11a1 on lesion expansion following *L. major* infection.

Slc11a1 is not expressed in M1/70-positive/iNOS-positive cells recruited to the liver following *L. donovani* infection

Phagocyte oxidase activity is known to be expressed in young monocytes recruited to sites of inflammation but not in the resident Kupffer cell population in the liver [40]. To determine whether Slc11a1 was preferentially expressed in Kupffer cells compared with fresh monocytes entering the liver after infection, we costained liver sections taken 2–4 days after *L. donovani* infection with monoclonal M1/70 recognizing CD11b (CR3) and a rabbit polyclonal anti-Slc11a1 Ab (**Fig. 7A**). No colocalized staining was observed, consistent with expression of Slc11a1 in the resident Kupffer cell population and not on freshly recruited M1/70-positive monocytes. Costaining of liver sections with rabbit polyclonal anti-iNOS Ab and rat monoclonal M1/70 demonstrated strict, colocalized staining of iNOS (Fig. 7B) in the M1/70-positive population (Fig. 7C). Slc11a1-positive cells in the liver were F4/80-positive (data not shown). Hence, Slc11a1 is expressed in resident Kupffer cells in the liver that do not express iNOS (our data) or phagocyte oxidase [40] activities.

DISCUSSION

Previous studies have demonstrated that regulation of *S. enterica* serovar Typhimurium and *L. donovani* infection in mice is divided into discrete phases, which rely on innate versus T cell-mediated immune responses [31, 41, 42]. In both cases, the *Nramp1*, formerly known as *Ity*, *Lsh*, or *Bcg* and now redesignated as *Slc11a1*, has its primary impact during the early innate phase(s) of infection [32, 33], and polymorphism at H-2 impacts on later T cell-mediated immune responses [39, 41]. The question we addressed was to what extent gp91phox and iNOS activities are required for these separate innate and acquired phases of the immune response to *S. enterica* serovar Typhimurium and *L. donovani*, focusing, in particular, on their role in Slc11a1-mediated resistance following the observations that gp91phox [10] and iNOS [8, 9, 15] expression and/or activities are differentially regulated in *Slc11a1*$^{+/+}$ and *Slc11a1*$^{mt/mt}$ macrophages in vitro. In addition, there was the specific hypothesis [20] that Fe^{2+} transported by Slc11a1 might interact directly with products of the oxidative burst to provide more potent antimicrobial radicals. Earlier studies of *S. enterica* serovar Typhimurium infection in *Cybb*$^{-/-}$ mice on a B6 *Slc11a1*$^{mt/mt}$ genetic background [43] had suggested some independence between oxidative burst-dependent host defenses, which are crucial in the first 24 h of infection, and *Slc11a1*, which extends its regulation of infection over the first ~7 days of infection [44]. This suggestion is confirmed here by our demonstration that *Slc11a1*$^{+/+}$ maintain equivalent advantage over *Slc11a1*$^{mt/mt}$ mice with or without the *Cybb*$^{-/-}$ gene disruption and *Cybb*$^{-/-}$ exerting its influence independently and earlier than *Slc11a1*. Similarly, earlier studies of *S. enterica* serovar Typhimurium [43] had suggested that iNOS activity was important later (post-day 7) in infection, again, independent of *Slc11a1* control, although this conclusion was, to some extent, serendipitous, as we show here that the B6 *Nos2A*$^{-/-}$ mice used [22], in fact, retained the *Slc11a1* wild-type allele from the 129 progenitor (Mastroeni and co-workers [43] did not Slc11a1-genotype the mice used in their experiments), and the 129Sv *Nos2A*$^{-/-}$ strain used [36] in that study is not a full, functional null but a hypomorph, as demonstrated by the presence of a full-length protein in Western blots using control-activated macrophage protein preparations (J. K. White and J. M. Blackwell, unpublished data). This is consistent with functional studies [45] demonstrating that this 129Sv *Nos2A*$^{-/-}$ line was leaky for NO production. Nevertheless, results obtained here using the full, functional *Nos2A* null, backcrossed onto equivalent B6/B10 *Slc11a1* wild-type and mt genetic backgrounds, support the conclusion that functional iNOS is not required for *Slc11a1*-mediated resistance over the first 7 days of a virulent *S. enterica* serovar Typhimurium C5 infection.

One problem in attempting to clarify the possible influence of gp91phox and iNOS on *Slc11a1*-mediated resistance in the context of *S. enterica* serovar Typhimurium infection is the acute and lethal nature of the infection and the short time-frame in which to examine the different phases of infection following administration of a virulent wild-type strain of the pathogen. In addition, the pleiotropic effects that Slc11a1 has on macrophage activation phenotypes in vitro mean that the same mechanisms might not be important in the in vivo regulation of different pathogens, which come under Slc11a1 control. In particular, we were keen to

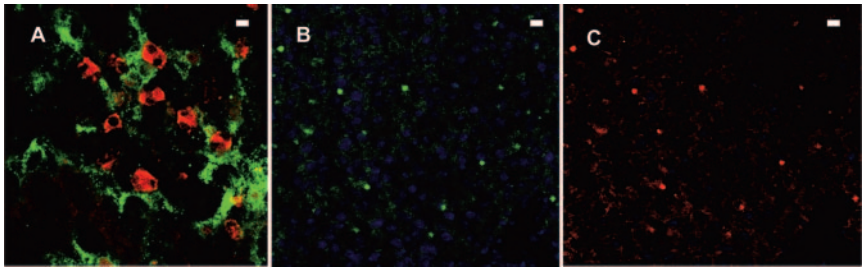

Fig. 7. Immunofluorescence staining of liver crystat sections from N20 mice 2–4 days after infection. (A) Representative field examined under high power with confocal laser attachment demonstrating no colocalization of Slc11a1 staining in resident Kupffer cells (green) with M1/70 staining on fresh infiltrating monocytes (red). Original bar = 10 μm. (B and C) Complete colocalization of M1/70 (green) and iNOS (red) in infiltrating monocytes with no iNOS staining of the resident Kupffer cell population. Original bars = 100 μm.

determine the influence of gp91phox and iNOS in Slc11a1 regulation of *L. donovani* infection, as both had been shown to be important in killing *L. donovani* in macrophages in vitro [16, 17]. Unlike *Salmonella* infection, *L. donovani* infection is not lethal in mice, and the more prolonged course of infection makes it a more tractable, experimental model in which to analyze the roles of gp91phox and iNOS in *Slc11a1*- and non-*Slc11a1*-regulated phases of infection. Using this model, we obtained clear evidence that neither gp91phox nor iNOS activity contributed to resistance to *L. donovani* infection over the first 15 days when Slc11a1 is known to exert maximal influence in liver and spleen [26, 33]. Our results differed from the earlier study of *L. donovani* infection using the same B6 ($Slc11a1^{mt/mt}$) background mice [23] in which an increase in parasite loads in the livers of $Cybb^{-/-}$ compared with $Cybb^{+/+}$ mice was reported at 2 weeks post-infection [46]. However, our study and the earlier study [46] concur on the observation that gp91phox has no influence over the acquired phase (>2 weeks) of *L. donovani* infection. Using a different source of $Nos2A^{-/-}$ mice on a mixed B6 × 129/Sv background [47], these authors also report [46] a more dramatic effect of iNOS deficiency on early (2 weeks) and acquired (>2 weeks) phases of *L. donovani* compared with our study in which iNOS had only moderate, organ-specific effects in the spleen at 50 days post-infection. Although the *Slc11a1* status of these mixed-background mice was not reported [46], it is possible, in this case, that the line had become fixed for the B6 $Slc11a1^{mt}$ allele and that the requirement for iNOS activity during the acquired phase of immunity is more critical in $Slc11a1^{mt/mt}$ mice than we have observed here in $Slc11a1^{+/+}$ mice. The results we obtained in our study with *L. donovani* were in stark contrast to *L. major* infection, where as reported previously [38], the fully functional $Nos2A^{-/-}$ gene disruption on a normally *L. major*-resistant B6/B10 background led to dramatic, early susceptibility and necessity for euthanasia within the first 21 days post-infection and where $Cybb^{-/-}$ also showed some influence on lesion size over the course of *L. major* infection. The smaller influence of $Cybb^{-/-}$ on *L. major* infection compared with $Nos2A^{-/-}$ is consistent with previous work [48], showing no effect of gp91phox on resolution of acute skin lesions and only a limited effect on containment of parasite in the draining lymph node. It is intriguing, however, that gp91phox was essential for clearance of *L. major* from the spleen [48]. These species-specific differences reflect the different cellular tropisms and pathologies caused by *L. donovani* and *L. major*. Studies in vitro [49] and in vivo [35] show that *L. major* has a preference for young, M1/70-positive monocytes, even when injected as amastigotes into mice i.v. and monitored in the liver [35], placing them in a cell population readily activated to express toxic antimicrobial iNOS activity. *L. donovani* amastigotes, conversely, preferentially infect resident tissue macrophages [35] and are found in the bone marrow in long-lived, sialoadhesin-positive, resident, stromal bone marrow macrophages [50]. This ability to target macrophage populations, which do not normally express potent antimicrobial iNOS or gp91phox activities, means that other mechanisms, such as those regulated more directly by Slc11a1, must be deployed by the host to control *L. donovani* infection. The ability of *L. donovani* to sequester into resident macrophage populations likely plays a major role in parasite persistence, with Slc11a1 possibly playing an important, long-term role in keeping this persistent parasite population in check. The species-specific differences in the behavior of leishmanial parasites in vivo and, in particular, the different mechanisms of antimicrobial activity associated with resistance and susceptibility to *L. donovani* and *L. major* have important implications for our understanding of human disease and the development of appropriate therapeutics.

To understand why neither gp91phox nor iNOS played a role in Slc11a1-mediated resistance in vivo, despite studies showing differential regulation of expression of activity of both in macrophages in vitro, we considered whether they were expressed in the same or different macrophage populations following infection in vivo. Although not formally re-examined here, we knew from earlier studies [40] that oxidative burst activity is not observed in the resident Kupffer cell population in the liver. What we examined specifically was which subpopulations of macrophage express Slc11a1 in the liver following *L. donovani* infection and whether this population expressed iNOS. Although we were unable to colocalize Slc11a1 and iNOS directly (as both were probed with rabbit Ab), we did demonstrate that iNOS and CD11b (recognized by the M1/70 Ab) were exclusively coexpressed in the fresh monocytes entering the liver after infection and that the M1/70 Ab showed no colocalization with the Slc11a1-positive, F4/80-positive, resident Kupffer cell population. Hence, we conclude that iNOS is not coexpressed in the same population of macrophages in the liver as Slc11a1 and similarly, by inference, that gp91phox and Slc11a1 are also unlikely to be coexpressed in the same macrophages in the liver. This is consistent with early studies [51–53] demonstrating that *Lsh/Ity* (=*Slc11a1*)-mediated resistance to infection was expressed in resident tissue macrophages, in Kupffer cells isolated ex vivo from livers of infected mice [51], or in liver, lung, or splenic tissue macrophages isolated and infected in vitro [52, 53] but not in less mature macrophages from the peritoneal cavity [53]. Similarly, mature bone marrow-derived macrophages expressed *Lsh*-mediated resistance to *L. donovani* infection in vitro, but immature macrophages kept cycling with growth factors did not [53]. This is consistent now with RNA and Ab studies demonstrating that bone marrow macrophages have to be matured and activated to express high levels of Slc11a1 [54] and our data here showing that Kupffer cells in the livers of *L. donovani*-infected mice express Slc11a1 but not iNOS or by inference, gp91phox. Overall, our results suggest that although Slc11a1 may influence iNOS and NADPH oxidase activity under conditions where macrophages are activated to coexpress Slc11a1/iNOS or Slc11a1/NADPH oxidase in vitro, this does not occur for all populations of macrophages examined in vitro and does not appear to occur to influence the course of infection in vivo.

Our failure to demonstrate a role for NADPH oxidase or iNOS activity in Slc11a1-regulated *S. enterica* serovar Typhimurium or *L. donovani* infection leads us to conclude that interaction between these molecular pathways is not involved in Slc11a1-mediated resistance to infection in vivo. Other explanations must be found to account for Slc11a1-mediated antimicrobial activity. Early studies concluded that Slc11a1 limits bacterial [55] or leishmanial [56] replication in vivo, rather than enhancing bacterial killing. More recent studies have focused on the influence of Slc11a1 on phagosome-lysosome fusion and maturation in macrophages in vitro [54, 57–60]. Functional Slc11a1 promotes pha-

gosome-lysosome fusion and maturation, leading to acidification of the pathogen-containing phagosome and creating conditions under which reduced pathogen replication can occur. This appears to be related directly to the divalent cation-transporting function of Slc11a1, as iron chelators restore recruitment of the mannose-6-phosphate receptor to *Salmonella* containing vacuoles in Slc11a1 mt macrophages, overcoming the ability of *Salmonella* to alter phagosome maturation [61]. This suggests a role for Slc11a1 in regulating the recently described, Toll-like receptor-triggered, inducible, phagosome-lysosome maturation pathway [62], perhaps accounting for the influence of Slc11a1 on antigen processing and presentation [63] and T helper cell type 1 (Th1)/Th2 bias in immune response to infection [64, 65] or vaccination [66].

In conclusion, our studies are important in demonstrating that observations made in vitro do not necessarily reflect the course of events in vivo. We demonstrate that iNOS and gp91phox are not important players in Slc11a1-mediated resistance to infection in vivo, fueling further research into the mechanisms by which this important gene influences susceptibility to infectious and autoimmune diseases.

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