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ORIGINAL INVESTIGATION

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Nramp1 gene affects selective early steps in macrophage-mediated anti-cryptococcal defense

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Abstract *Cryptococcus neoformans* is an opportunistic fungus responsible for severe and often recurrent meningoencephalitis in immunodepressed patients. Initial evidence suggests that *C. neoformans* is a facultative intracellular pathogen; however, the strategies by which *C. neoformans* undergoes survival and eventually proliferation have not been elucidated. We investigated the role of *Nramp1* gene in macrophage-mediated anti-cryptococcal defense. Using cell lines expressing the functional, mutated or knockout gene, it was established that *Nramp1* (1) is not involved in the phagocytic event, (2) influences anti-cryptococcal activity in the early steps but not at later times, and (3) is unrelated to the biomolecular pathways through which *C. neoformans* impairs macrophage secretory response. Although the functional role of *Nramp1* is still far from being elucidated, the present data add insight into its involvement in macrophage-mediated antimicrobial defense, particularly in the initial steps allowing *C. neoformans* growth inhibition.

Keywords *Cryptococcus neoformans* · Macrophages · *Nramp*1 gene · Anti-cryptococcal activity

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Introduction

In mice, resistance or susceptibility to infections with intracellular parasites is determined by the locus *bcg,* also known as *ity* or *lsh,* present in two allelic forms (*bcg*^{s} and *bcg*^{r}) within chromosome 1. Infections controlled by the *bcg* locus include several mycobacterial species *(Mycobacterium bovis, M. avium, M. lepraemurium),* as well as *Salmonella typhimurium* and *Leishmania donovani* [15, 31]. The genetic control results in a rapid microbial replication during the early phase of infection in susceptible *(bcg*^{s}*)* mice, as opposed to the absence of multiplication in resistant *(bcg*^{r}*)* animals (for review see [31]). The cell population(s) responsible for phenotypic expression of *bcg* is bone marrow derived, resistant to radiation and sensitive to the phagocyte poison silica [31]. Furthermore, macrophages obtained from *bcg*^{r} and *bcg*^{s} mice show different capacities to restrict growth of *Mycobacteria*, *Salmonella* and *Leishmania* in vitro. Overall, these results indicate that the macrophage is the cell type expressing the genetic difference at the *bcg* locus, which in turns reflects different degree of bactericidal or bacteriostatic activity. Using positional cloning, a candidate for *bcg* gene, designed *Nramp1* (natural resistance-associated-macrophage protein 1) has been identified [34]. Haplotype mapping [22], gene targeting [35], transgenic mice technology [16] and biallelic cDNA transfection [4] provided direct proof that *Nramp1* and *bcg*-*ity*-*lsh* are the same gene. *Nramp1* encodes a highly hydrophobic protein of 65 kDa, with characteristics of an integral membrane protein, including 12 putative transmembrane (TM) domains, a glycosylated extracellular loop and several phosphorylation sites [15]. Sequencing of the *Nramp1* mRNA from *bcg*^{r} and *bcg*^{s} inbred strains revealed that susceptibility to infection is associated with a nonconservative glycine to aspartic acid substitution at position 169 (G 169 D), within the fourth predicted TM domain of the protein [22]. Although the exact function of the Nramp1 protein has not been

clarified yet [9], homology with the yeast membrane transporters, SFM1 and SFM2 (from *Saccharomyces cerevisiae*), suggests that the *Nramp1* protein may be involved in the transport of divalent cations, such Mn^{2+}, Zn^{2+} or Fe^{2+}, across the membrane [33]. The intracellular localization of the *Nramp1* protein has been analyzed by immunofluorescence and confocal microscopy and by biochemical fractionation. *Nramp1* has been found not to be expressed at the cytoplasmic membrane but rather localized to the late endocytic vesicles (late endosome/lysosome) of resting macrophages in a Lamp1 (lysosomal-associated membrane protein 1)-positive compartment [18]. Double immunofluorescence studies and direct purification of latex bead-containing phagosomes demonstrated that upon phagocytosis, *Nramp1* is recruited to the membrane of the phagosome and remains associated with this structure during its maturation to phagolysosome. The targeting of *Nramp1* from endocytic vesicles to the phagosomal membrane supports the hypothesis that *Nramp1* controls the replication of intracellular parasites by altering the intravacuolar environment of the microbe-containing phagosome [18]. A second mammalian *Nramp* gene, *Nramp*2, which encodes a highly homologues protein, has been isolated [17]. However, as opposed to the phagocyte-specific *Nramp1* counterpart, *Nramp2* is expressed in most tissues. Database searches and additional cloning experiments have shown that *Nramp* comprises a family of proteins that are extremely conserved throughout the evolution. Members of the *Nramp* protein family can be found in such distinct evolutionary species as bacteria, fungi, plants, insects, worms, birds, and mammals, including humans [5, 9, 10, 19, 29, 37].

Cryptococcus neoformans is the causative agent of cryptococcosis, a life-threatening fungal infection in immunodepressed patients [24]. Acquired from the environment by inhalation [11], most primary infections are asymptomatic in immune competent hosts [20]. In patients with AIDS and other immunocompromised patients, *C. neoformans* targets the central nervous system causing (often recurrent) meningoencephalitis [25]. Most clinical cases of relapses result from reactivation of the original strain [14], thus implying that *C. neoformans* may adapt to persist in tissue(s); the mechanisms by which it resists an immune response and eventually undergoes reactivation are not yet understood. Fungal molecular plasticity, referred as microevolution, has recently been documented and likely accounts for cryptococcal dormancy and subsequent reactivation in the recurrences (Blasi et al., submitted). A recent study suggests that *C. neoformans* is a facultative intracellular pathogen *in vivo*, capable of surviving inside lung macrophages, where intracellular replication is then associated with host cell toxicity and profuse accumulation of fungal polysaccharides [12].

The purpose of this study was to compare macrophages with functional, mutated or deleted *Nramp1* gene for capacity in handling cryptococcal infection.

Materials and methods

Reagents

Recombinant murine interferon-γ (IFN-γ) was obtained from PharMingen (San Diego, Calif.). Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0128:B 12) and chloroquine (CQ) were purchased from Sigma (St. Louis, Mo.).

Cell lines

The two sets of macrophage cell lines employed in this study were: (1) the bone marrow macrophage cell lines 129.1 (derived from 129/J mice carrying the wild-type, functional *Nramp1* gene) and 129.KO (derived from 129/*Nramp1* gene knockout mice, with genetically disrupted *Nramp1* gene) [38]; (2) the microglial cell lines RR4 [derived from C57Bl/10.A *(bcg^r)* carrying the *Nramp1* wild type] and RR8 [derived from C57Bl/10.A (*bcg^s)* carrying the D169 mutated *Nramp1* gene] [27]. The cell lines had been obtained by immortalization of primary cultures via introducing retroviral oncogenes. They expressed and retained the phenotypical peculiarities of the original cultures and have been extensively used for functional and molecular studies on macrophages [1, 3, 6, 7, 26, 27, 38].

Cells were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT), gentamycin (50 μg/ml) and L-glutamine (2 μM) (complete medium). Biweekly, cells were detached by vigorous shaking and were replaced by fresh cultures. For clarity and in accordance with the literature [4, 16, 22, 27], 129.1 and RR4 cells, carrying the wild type gene, are hereafter referred to as *Nramp1*$^+$, and 129.KO and RR8, carrying the mutated or deleted gene, as *Nramp1*$^-$ cells.

Microorganisms

C. neoformans strain ATCC 11240 was maintained by biweekly passages on Sabouraud dextrose agar plates, kept at room temperature. Yeast cells were harvested from the agar plates, washed twice in saline by low-speed centrifugation, and diluted to the appropriated concentration in complete medium prior to use in the *in vitro* assays. Opsonization of *C. neoformans* was performed by 60-min preincubation in mouse serum (10^8 yeast cells/500 μl) obtained from mice infected 8–10 weeks earlier with sublethal doses of *C. neoformans*. Yeast cells were then washed twice in saline prior to be used in the assays.

Phagocytosis assay

C. neoformans yeast cells (10^7 cells/ml) were incubated with the macrophages (10^6 cells/ml) for 2 h at 37°C. The excess of *C. neoformans* was then removed by centrifugation of the cell suspension on a Ficoll cushion at 300 *g* for 10 min. Cells at the interface were recovered and washed. *C. neoformans* uptake was directly evaluated in Giemsa-stained cytospin preparations. A minimum of 200 cells were scored, and any cells containing one or more particles were counted as phagocytic. The phagocytosis index was calculated as total number of phagocytosed yeasts/total number of phagocytic cells.

Measurement of anti-cryptococcal activity

Macrophages were plated (5×10^5 cells/ml) in 96-well plates (Corning Glass Works, Corning, N.Y.) with or without IFNγ (100 U/ml;18-h incubation) with or without CQ (10 μmol/ml; 1 h)

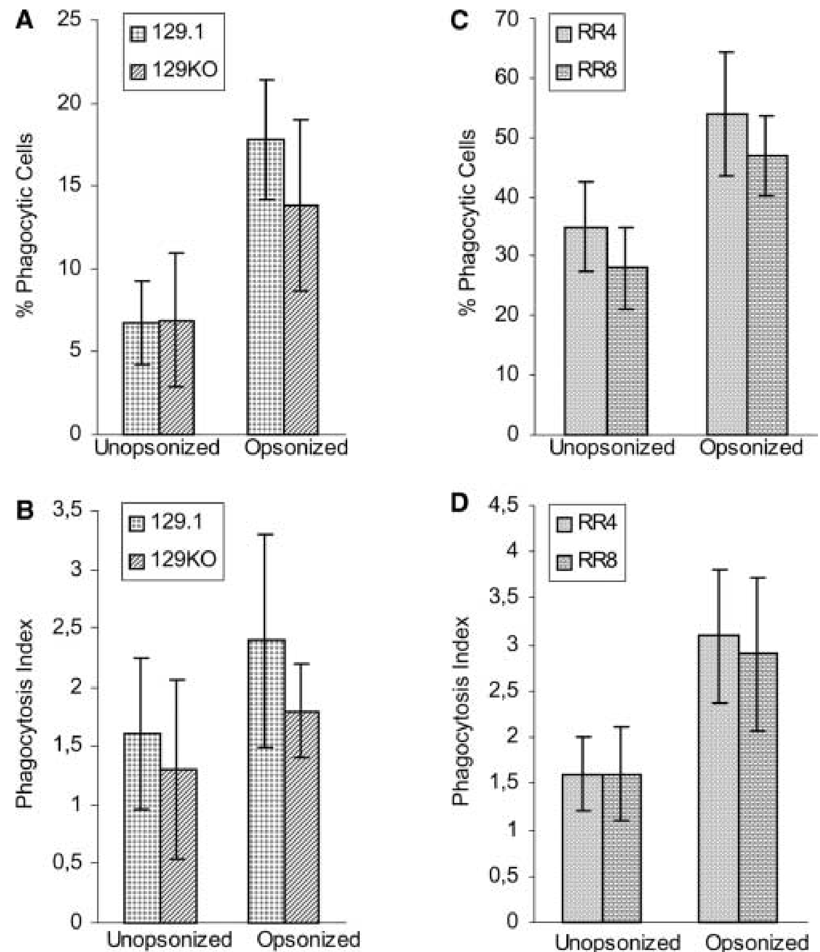


Fig. 1A–D Phagocytic activity of *Nramp1*$^+$ and *Nramp1*$^-$ cells against unopsonized and opsonized *C. neoformans*. Bone marrow macrophages (**A**, **B**), 129.1 (*Nramp1*$^+$) and 129. KO (*Nramp1*$^-$) cells, and brain macrophages (**C, D**), RR4 (*Nramp1*$^+$) and RR8 (*Nramp1*$^-$) cells, were exposed to *C. neoformans* at a macrophage: *C. neoformans* ratio of 1:10 for 2 h; *C. neoformans* was used unopsonized or opsonized. The percent of phagocytic cells (**A**, **C**) and the phagocytosis index (**B**, **D**) were evaluated in Giemsa-stained cytospin preparations. Values at the mean ± SD of three independent experiments

at 37°C in 5% CO_2. *C. neoformans* was then added at a macrophage:*C. neoformans* ratio of 10:1. After 4, 8 and 24 h, Triton X-100 (0.1% final concentration) was added to the wells and appropriate dilutions were plated on Sabouraud dextrose agar. The colonies were counted after 48–72 h of incubation at room temperature. Control cultures consisted of *C. neoformans* incubated without effector cells. Results were expressed as percentage of colony-forming units (cfu) inhibition according to the following formula:

$$\text{cfu inhibition} = 100 - \text{cfu experimental groups/cfu control cultures} \times 100$$

Measurement of cytokine production

To determinate cytokine production, macrophages (10^6 cells/ml) were exposed to *C. neoformans* (at macrophage:*C. neoformans* ratios of 1:10 and 1:1) for 18 h at 37°C; LPS (1 μg/ml) was added for additional 3 h. Supernatants were then collected and assayed for cytokine levels. Interleukin1α (IL-1) and tumor necrosis factorα (TNFα) were tested with PharMingen kits and IL-6 with Biosource according to the manufacturer's recommendations.

Statistical analysis

The significance of the data was evaluated using the Student's *t-test*. Each experiment was repeated three to seven times. Values in the figures represent the mean ± SD of three independent experiments, with the exception of Fig. 6, where values are the mean ± SD of four determinations observed in two independent experiments.

Results

To investigate the role of *Nramp1* gene in the accomplishment of anti-cryptococcal activity by macrophages, cell lines representative of the *Nramp1* functional (129.1 and RR4 cells), mutated (RR8 cells) or deleted (129.KO cells) genotype were tested for phagocytic activity against *C. neoformans*. The percent of phagocytic cells and the phagocytosis index were evaluated in each cell population using unopsonized and opsonized *C. neoformans* in a 2-h assay. As shown in Fig. 1, 129.1 and 129.KO cells exhibited low and comparable levels of phagocytic activity against unopsonized *C. neoformans* (Fig. 1A); when tested against opsonized *C. neoformans,* the phagocytic activity consistently augmented in both cell lines, the increase being slightly more evident in the 129.1 than in 129.KO cells. The phagocytosis index was also comparable between the two cell lines and slightly increased when using opsonized *C. neoformans* (Fig. 1B).

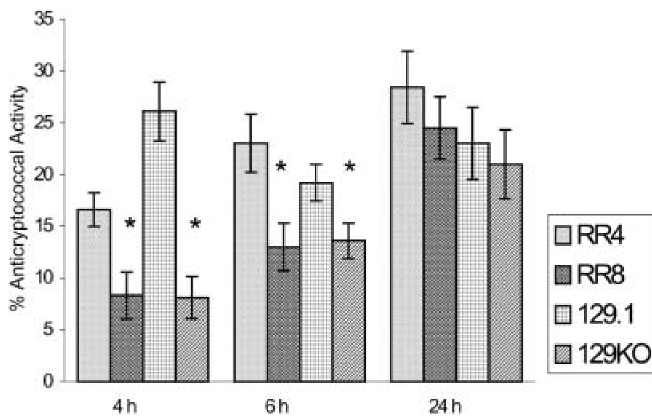


Fig. 2 Kinetic of anti-cryptococcal activity by *Nramp1*$^{+}$ and *Nramp1*$^{-}$ cells against *C. neoformans*. Bone marrow macrophages, 129.1 (*Nramp1*$^{+}$) and 129.KO (*Nramp1*$^{-}$) cells, and brain macrophages, RR4 (*Nramp1*$^{+}$) and RR8 (*Nramp1*$^{-}$) cells, were exposed to *C. neoformans* at a macrophage:*C.neoformans* ratio of 10:1 for 4, 6, or 24 h. The anti-cryptococcal activity was evaluated as % cfu inhibition according to the formula depicted in the Materials and methods section. Values represent the mean ± SD of three independent experiments. *$P < 0.01$, *Nramp1*$^{+}$ versus *Nramp1*$^{-}$ cells (*cfu* colony-forming unit)

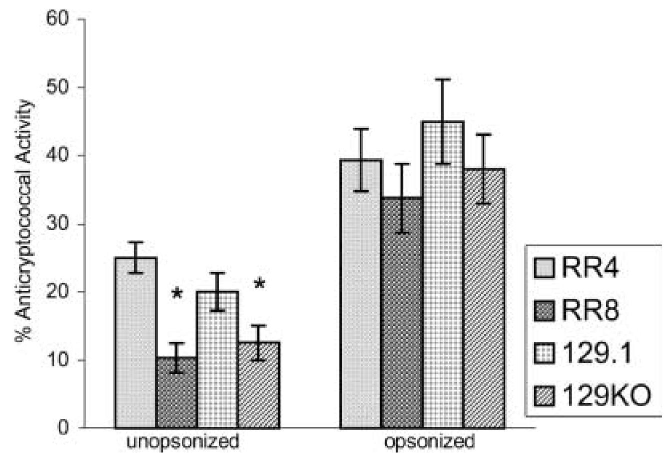


Fig. 3 Anti-cryptococcal activity of *Nramp1*$^{+}$ and *Nramp1*$^{-}$ cells against unopsonized and opsonized *C. neoformans*. Bone marrow macrophages, 129.1 (*Nramp1*$^{+}$) and 129.KO (*Nramp1*$^{-}$) cells, and brain macrophages, RR4 (*Nramp1*$^{+}$) and RR8 (*Nramp1*$^{-}$) cells, were exposed to *C. neoformans* at a macrophage:*C. neoformans* ratio of 10:1 for 6 h. *C. neoformans* opsonization was achieved by 60-min preincubation in mouse hyperimmune serum. The anti-cryptococcal activity was evaluated as % cfu inhibition. Values represent the mean ± SD of three independent experiments. *$P < 0.01$, *Nramp1*$^{+}$ versus *Nramp1*$^{-}$ cells

Figure 1C, D shows data relative to the phagocytic activity of RR4 and RR8 cells. Although the microglial cells were more effective than their bone marrow counterparts, no significant differences were detectable between RR4 and RR8 in terms of percent phagocytic cells (Fig. 1C) and phagocytosis index (Fig. 1D), regardless of whether unopsonized or opsonized *C. neoformans* was used.

To compare the anti-cryptococcal activity of *Nramp1*$^{+}$ and *Nramp1*$^{-}$ cells, kinetic experiments were performed at a macrophage:*C.neoformans* ratio of 10:1. The results obtained (Fig. 2) indicated that 129.1 and RR4 were significantly more active than the correspondent counterparts, 129.KO and RR8, in the 4 and 6 h assays. Differently, comparable levels of activity among *Nramp1*$^{+}$ and *Nramp1*$^{-}$ cells were observed in the 24 h assay. Because of the differences observed in the short-term assays, the anti-cryptococcal activity of *Nramp1*$^{+}$and *Nramp1*$^{-}$ cells was tested in the 6 h assay, using unopsonized and opsonized *C. neoformans*. Differences were confirmed between 129.1 and 129.KO and also between RR4 and RR8, when unopsonized *C. neoformans* were used. As predicted, all the cell lines were significantly more effective when using opsonized *C. neoformans*; moreover, under those experimental conditions, the anti-cryptococcal activities of *Nramp1*$^{+}$ and *Nramp1*$^{-}$ cells reached comparably high levels, irrespective of the genetic background (Fig. 3).

Since IFNγ and CQ are known to be stimulatory for macrophage functions [7, 23], we investigated the effects of both agents on the phagocytic potential of the *Nramp1*$^{+}$and *Nramp1*$^{-}$ cell lines. According to previously established protocols [7, 23, 38], 129.1 and 129.KO cells were exposed to IFNγ or CQ for 18 and 1 h, respectively, prior to be tested in the phagocytosis assay. It was found that IFNγ consistently enhanced the phagocytic activity of both cell lines to a similar extent, while CQ was totally ineffective (Fig. 4). In parallel experiments, we tested the anti-cryptococcal activity of *Nramp1*$^{+}$and *Nramp1*$^{-}$ cells following exposure to IFNγ or CQ. Both agents caused a major increase in the activity of all the cell lines studied. Interestingly, the degree of enhancement was consistently higher in *Nramp1*$^{+}$ than in *Nramp1*$^{-}$ cells (Fig. 5), when testing both bone marrow and brain-derived macrophages.

We have previously shown that *C. neoformans* is capable of impairing the secretory functions of macrophages by affecting TNFα secretion in response to LPS [3]. To ascertain the possible involvement of *Nramp1* gene in the phenomenon, 129.1 and 129.KO cells were exposed to *C. neoformans* and then stimulated with LPS. The levels of cytokine production were evaluated in cell-free supernatants by ELISA. As depicted in Fig. 6, both cell lines constitutively produced appreciable levels of IL-1 and TNFα. *C. neoformans* infection did not alter such levels, while LPS treatment significantly enhanced cytokine production, both in 129.1 and 129.KO cells. When LPS stimulation was performed on cells that had been previously infected with *C. neoformans*, both IL-1 and TNFα induction was impaired in 129.1 and in 129.KO cells. The inhibitory effect was similar among different cell lines and was dependent on the macrophage:*C.neoformans* ratios employed. The only significant difference between 129.1 and 129.KO was observed in TNFα production. Finally, while undetectable in control and infected cultures (Fig. 6), IL-6 was highly produced upon LPS stimulation both in 129.1 and 129.KO cells. LPS treatment of infected cells did not

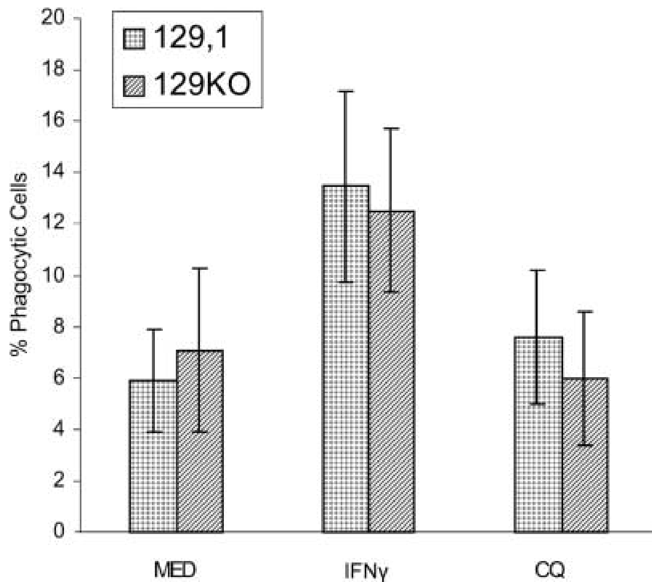
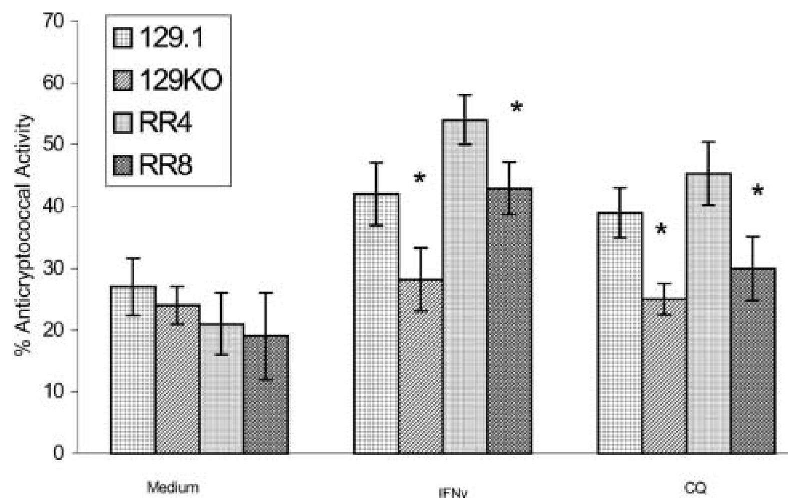


Fig. 4 Phagocytic activity of *Nramp1*$^+$ and *Nramp1*$^-$ cells preincubated with or without IFNγ or CQ and then exposed to *C. neoformans*. Bone marrow macrophages, 129.1 (*Nramp1*$^+$) and 129.KO (*Nramp1*$^-$) cells, were exposed to 100 U/ml IFNγ or 10 μmol/ml CQ for 18 and 1 h, respectively, prior to be tested in the phagocytosis assay. Then, cells were infected with *C. neoformans* at a macrophage:*C. neoformans* ratio of 1:10 for 2 h. The percent of phagocytic cells were evaluated in Giemsa-stained cytospin preparations. Values represent the mean ± SD of three independent experiments (*CQ* chloroquine)

affect IL-6 production, when either 1:10 or 1:1 macrophage:*C. neoformans* ratio was used.

Fig. 5 Anti-cryptococcal activity of *Nramp1*$^+$ and *Nramp1*$^-$ cells preincubated with or without IFNγ or CQ and then exposed to *C. neoformans*. Bone marrow macrophages, 129.1 (*Nramp1*$^+$) and 129.KO (*Nramp1*$^-$) cells, and brain macrophages, RR4 (*Nramp1*$^+$) and RR8 (*Nramp1*$^-$) cells were exposed to 100 U/ml IFNγ or 10 μmol/ml CQ for 18 and 1 h respectively prior to be tested in the anti-cryptococcal assay. Then, cells were infected with *C. neoformans* at a macrophage:*C. neoformans* ratio of 10:1 for 24 h. The anti-cryptococcal activity was evaluated as % cfu inhibition. Values represent the mean ± SD of three independent experiments. *$P < 0.01$, *Nramp1*$^+$ versus *Nramp1*$^-$ cells



Overall, these findings indicate that *Nramp1* gene affects selective early steps in macrophage-mediated anti-cryptococcal defenses.

Discussion

The present report provides evidence that the *Nramp1* genotype influences to some extent the immunological response of macrophages to *C. neoformans*. The importance of the *Nramp1* gene in murine resistance/susceptibility to several infections has been documented. Targeted gene disruption and reconstitution approaches formally prove that *Nramp1* is involved in the early nonimmune phase of infection with intracellular parasites [16, 35]. General consensus points to the mature macrophage as the cell population involved in phenotypic expression of the gene, whose product is recruited to the membrane of the microbe-containing phagosome [18]. Such a subcellular localization, together with the high homology of *Nramp1* protein with a number of eukaryotic and procaryotic membrane transport proteins, supports the hypothesis that *Nramp1* affects the replication of intracellular parasites by altering the intravacuolar environment where the pathogen is confined. Initial evidence indicates that *Nramp1* also influences macrophage response to fungi, particularly to the dimorphic opportunistic pathogen *Candida albicans* [26]. Overall, *Nramp1* gene affects macrophage potential against antigenically and taxonomically unrelated microbes, thus implying that the antimicrobial devises depending on intact *Nramp1* have a wide spectrum of action and/or that *Nramp1* protein has pleiotropic effects.

By means of cell lines genetically differing for *Nramp1* gene (wild type, mutated or disrupted), we show here that macrophage interplay with *C. neoformans* depends to some extent on *Nramp1* genotype/phenotype.

First, phagocytosis appears uncoupled with *Nramp1*, in that *Nramp1*$^+$ and *Nramp1*$^-$ macrophages ingest *C. neoformans* with comparable efficacy; both the

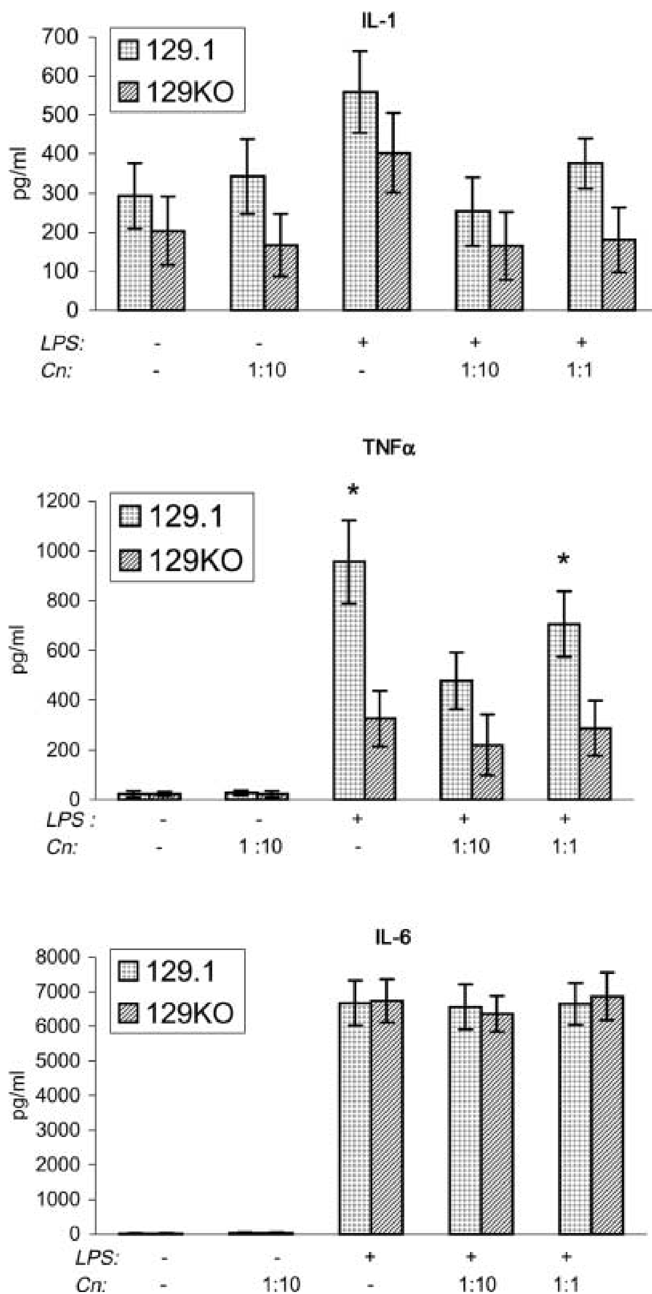


Fig. 6 Secretory activity of *Nramp1*$^+$ and *Nramp1*$^-$ cells. Bone marrow macrophages, 129.1 (*Nramp1*$^+$) and 129.KO (*Nramp1*$^-$) cells, were exposed to *C. neoformans* at the macrophage: *C. neoformans* ratio of 1:10 or 1:1 for 18 h, and then stimulated with 1 μg/ml LPS for additional 3 h. The levels of cytokine production were evaluated in cell-free supernatants by ELISA. Values represent the mean ± SD of four determinations obtained in two independent experiments. *$P < 0.01$, *Nramp1*$^+$ versus *Nramp1*$^-$ cells

percent of phagocytic cells and the phagocytosis index are in fact similar. As expected [2], the phagocytic activity is up-regulated by opsonization of the microorganism; again, no differences have consistently been observed between *Nramp1*$^+$ and *Nramp1*$^-$ cells. This is in line with previous data relative to another fungal pathogen [26], showing that *Nramp1* does not influence the phagocytic activity of macrophages, namely receptor-like structures and/or membrane-associated events allowing microbial uptake are not controlled by the *Nramp1* gene. This conclusion applies to both constitutive and IFNγ-induced phagocytosis, as we show that IFNγ stimulatory effects are comparable both in *Nramp1*$^+$ and *Nramp1*$^-$ cells.

Second, the anti-cryptococcal activity of murine macrophages is influenced by *Nramp1* genotype, provided that short-term assays are employed. *Nramp1*$^+$ cells are significantly more effective against *C. neoformans* than *Nramp1*$^-$ cells in the 4- and 6-h assays. Thus, apart from phagocytosis, other antimicrobial mechanisms are differently accomplished in macrophages according to the *Nramp1* genotype and influence to a different extent cryptococcal survival/growth. Nevertheless, the functional difference between *Nramp1*$^+$ and *Nramp1*$^-$ cells in terms of anti-cryptococcal activity disappears at later time points (24-h assay). This implies that additional antimicrobial pathways, apparently *Nramp1* independent, are pursued by macrophages in the long-term assay and guarantee for fulfillment of comparable effectiveness, irrespective of *Nramp*1 genotype. Thus, macrophages accomplish anti-cryptococcal activity through two mechanisms, distinguishable for dependency on *Nramp1* genotype, one being involved in the early and the other in the late phase of macrophage-*C. neoformans* interaction. Moreover, the present data show that *Nramp1*$^+$ and *Nramp1*$^-$ cells, although functionally different from each other when tested against unopsonized fungi, become comparably effective against opsonized *C. neoformans*. This implies that the antimicrobial mechanisms employed against unopsonized but not opsonized fungi are *Nramp1* dependent. Thus, we speculate that *Nramp1* is involved in the early events of macrophage-*C. neoformans* interaction; additional and/or alternative mechanisms occur later on and are pursued by phagocytes provided that opsonized fungi have been ingested and/or enough time for killing (24 h) is given. Another explanation for the data reported here is that *C. neoformans* employs a novel strategy to allow its own survival/replication within macrophages. In this respect, several pathogens are known to evade or utilize the phagocytic process of macrophages to survive and replicate inside host cells [28, 30, 32]. According to the present data, we postulate that *C. neoformans* is capable of influencing macrophage programs to such an extent that the functional advantage of being *Nramp1*$^+$ macrophages is eventually lost with time. This speculation is supported by the recent demonstration that *C. neoformans* harbored within macrophages releases and spreads around fungal products, such a glucuronoxilomannan [13], whose potent inhibitory effects have been described [3]. According to our hypothesis, we assume that following the internalization process by macrophages, *C. neoformans* localizes in phagosomes, whose functional maturation is in turn impaired; thus,

Nramp1 contribution in antifungal defenses is eluded. Although fully speculative, this idea is supported by in vivo data showing no consistent differences between *Nramp1*$^{+}$ and *Nramp1*$^{-}$ mice in handling cryptococcal infection (data not shown).

Among its pleiotropic effects, IFNγ is known to be a potent stimulus for macrophages whose effector and secretory functions are strongly enhanced [7, 27, 39]. With a similar efficacy [23], CQ enhances anti-cryptococcal activity of murine macrophages, through mechanisms poorly understood but likely involving interference with phagosomal acidification [36]. The fact that both IFNγ and CQ are more effective in enhancing anti-cryptococcal activity of *Nramp1*$^{+}$ than *Nramp1*$^{-}$ cells suggests the involvement of *Nramp1* in the IFNγ- or CQ-induced macrophage activation.

Third, the deleterious effects of *C. neoformans* on macrophage response to LPS are evident in both *Nramp1*$^{+}$ and *Nramp1*$^{-}$ cells, whose IL-1 and TNFα production is impaired. In contrast, IL-6 remains unaffected in both cell types. From here, the conclusion that the biomolecular pathway(s) through which *C. neoformans* impairs macrophage response to LPS is *Nramp1* unrelated. This result was somehow expected, since it is known [6] that macrophage secretory response to fungi may occur without ingestion, thus implying that *Nramp1*-expressing intracellular compartment(s) is not involved. Finally, it is worth noting that, although immortalized via an oncogene carrying retrovirus [27, 38], the cell lines used in these studies consistently retain the phenotype of the primary macrophage cultures both at the functional and molecular level [1, 3, 6, 7, 26, 27, 38], thus arguing on the accuracy and usefulness of the continuous cell line model employed.

In conclusion, we provide evidence that the interaction between macrophages and the opportunistic pathogen *C. neoformans* is partially controlled at the genetic level; in particular, selective early steps in macrophage-mediated defenses are dependent on functional *Nramp1* gene.

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