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Neospora caninum excreted/secreted antigens trigger CC-chemokine receptor 5-dependent cell migration

Tiago W.P. Mineo [a,b,1,*], Carlo J.F. Oliveira [a,c,1], Deise A.O. Silva [b], Leandro L. Oliveira [a], Antônio R. Abatepaulo [a], Dâmaso P. Ribeiro [b], Beatriz R. Ferreira [d], José R. Mineo [b], João S. Silva [a]

[a] Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, SP, Brazil
[b] Institute of Biomedical Sciences, Federal University of Uberlândia, Av. Pará 1720, Uberlândia 38400-902, MG, Brazil
[c] Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Room 2E32D, Rockville, MD 20852, USA
[d] Department of Maternal-Child Nursing and Public Health, School of Nursing of Ribeirão Preto, USP, Avenida Bandeirantes 3900, 14040 902 Ribeirão Preto, SP, Brazil

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ABSTRACT

Neospora caninum, the causative agent of neosporosis, is an obligate intracellular parasite considered to be a major cause of abortion in cattle throughout the world. Most studies concerning *N. caninum* have focused on life cycle, seroepidemiology, pathology and vaccination, while data on host–parasite interaction, such as host cell migration, mechanisms of evasion and dissemination of this parasite during the early phase of infection are still poorly understood. Here we show the ability of excreted/secreted antigens from *N. caninum* (*Nc*ESAs) to attract monocytic cells to the site of primary infection in both in vitro and in vivo assays. Molecules from the family of cyclophilins present on the *Nc*ESAs were shown to work as chemokine-like proteins and *Nc*ESA-induced chemoattraction involved G_i protein signaling and participation of CC-chemokine receptor 5 (CCR5). Additionally, we demonstrate the ability of *Nc*ESAs to enhance the expression of CCR5 on monocytic cells and this increase occurred in parallel with the chemotactic activity of *Nc*ESAs by increasing cell migration. These results suggest that during the first days of infection, *N. caninum* produces molecules capable of inducing monocytic cell migration to the sites of infection, which will consequently enhance initial parasite invasion and proliferation. Altogether, these results help to clarify some key features involved in the process of cell migration and may reveal virulence factors and therapeutic targets to control neosporosis.

1. Introduction

Neospora caninum, the causative agent of neosporosis, is an obligate intracellular apicomplexan parasite predominantly known for causing abortion and neonatal mortality in cattle and severe neuromuscular disease in dogs. Since it was first identified in 1988 (Dubey et al., 1988a,b), this protozoan has emerged as one of the most important etiological agents of abortion in cattle throughout the world (Innes et al., 2005; Dubey and Lindsay, 2006; Dubey et al., 2007). To date, in South America, the U.S.A., the European Union and New Zealand, neosporosis is reported as the leading cause of abortions in cattle (Anderson et al., 1997; Hemphill and Gottstein, 2000; Moore, 2004; Dubey and Lindsay, 2006; Dubey et al., 2007). Intermediate hosts of the parasite—for example, cattle—may acquire infection vertically or horizontally by exposure and ingestion of oocysts excreted by canine definitive hosts. In the initial phase of infection, parasites invade host cells including immune cells, disseminate to multiple organs and transform into the rapidly proliferating tachyzoite stage. The stress caused by the host immune response triggers stage conversion in slowly proliferating *N. caninum* bradyzoites, a quiescent stage of the parasite. When immunocompetence is impaired, as in the case of pregnancy, bradyzoites reactivate and differentiate, resulting in tachyzoite parasitemia, lesions, infection of the fetus and abortion (Hemphill et al., 2006).

While many studies of *N. caninum* have demonstrated important aspects of the life cycle, seroepidemiology, pathology and vaccination, data on host–parasite interaction, such as host cell migration and the mechanisms used by *N. caninum* to modulate immune cells as monocytes during the early phase of infection are still poorly understood. It is well known that a rapid recruitment of monocytes to the site of an infection can potentially bring significant support to the innate immune response against foreign pathogens. However, dendritic cells (DC) can transport intracellular pathogens such as *Listeria monocytogenes*, *Leishmania major*, *Mycobacterium tuberculosis*,

* Corresponding author. Address: Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, SP, Brazil. Tel.: +55 34 3218 2058; fax: +55 34 3218 2333.
E-mail address: tiago.mineo@pq.cnpq.br (T.W.P. Mineo).
[1] These authors contributed equally to this work.

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Salmonella typhimurium and *Toxoplasma gondii* away from the site of primary infection and help their propagation inside the host (Moll et al., 1993; Teitelbaum et al., 1999; Pron et al., 2001; Rescigno et al., 2001a,b; Lambert et al., 2006).

As *N. caninum* is an intracellular protozoan and can infect different immune cell types including natural killer (NK) cells, macrophages and DC (Boysen et al., 2006; Hemphill et al., 2006; Strohbusch et al., 2009), we hypothesized that the parasite could modulate cell migration in order to enhance invasion and proliferation during early phases of infection. To study the interaction between monocytes and *N. caninum* and determine the cell migratory consequences of the exposure to the parasite and its antigens, we chose an i.p. infection model in mice so that we could precisely control the delivery of parasites or antigens and evaluate early cell recruitment at the site of challenge. Moreover, using murine bone marrow-derived dendritic cells (BMDC) as a monocytic cell model, we also analyzed the capacity of *N. caninum* and their excreted/secreted antigens (*Nc*ESAs) to modulate this process.

2. Materials and methods

2.1. Animals

Six- to 8-week-old CCR5$^{-/-}$ and wild-type (WT) C57BL/6 background mice were bred and maintained at the animal facilities of the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, USP (Ribeirão Preto, Brazil), with food and water ad libitum. Maintenance and care of these animals complied with the guidelines of the Laboratory Animal Ethics Committee from the Institution. Animal euthanasia was performed in accordance with international welfare grounds, according to the American Veterinary Medical Association Guidelines on Euthanasia (2007).

2.2. In vitro N. caninum maintenance

Neospora caninum tachyzoites (NC-1 isolate) were maintained by serial passages in Vero cells cultured in RPMI 1640 medium (Gibco-BRL Life Technologies, Grand Island, NY, USA) supplemented with 2 mM glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 atmosphere at 37 °C. Parasite suspensions were obtained as previously described (Silva et al., 2007). Briefly, tachyzoites were harvested by scraping off the cell monolayer 2–3 days p.i. (approximately 80% lysis), passed through a 26-gauge needle to lyse any remaining intact host cells and centrifuged at low speed (45*g*) for 1 min at 4 °C to remove host cell debris. The supernatant containing parasite suspension was collected and then washed twice (1,000*g*, 10 min, 4 °C) in PBS. The resulting pellet was resuspended in PBS for experimental infections and antigen preparation. Parasite viability was assessed by Trypan blue staining and all experiments yielded over 95% of live tachyzoites.

2.3. Antigen preparation

Neospora caninum lysate antigen (NLA) was prepared as previously described (Silva et al., 2007). The parasite suspension was treated with protease inhibitors and lysed by freeze–thaw cycles followed by ultrasonication on ice. The presence of probable live parasites was monitored by light microscopy under Trypan blue staining. After centrifugation (10,000*g*, 30 min, 4 °C), supernatant was collected, filtered through a 0.22-μm membrane and its protein concentration determined (Lowry et al., 1951). Different batches were done for NLA preparation and pooled together to obtain the required protein concentration. NLA aliquots were stored at −20 °C until used.

Preparation of *Nc*ESA was carried out as described elsewhere (Ribeiro et al., 2009). Freshly liberated NC-1 tachyzoites (10^8 parasites/ml) were washed twice in PBS, resuspended in Hank's saline solution and incubated for 30 min at 37 °C at mild agitation. Parasites were then centrifuged (720*g*, 10 min, 4 °C), and the supernatant was collected and again centrifuged (10,000*g*, 30 min, 4 °C). The final supernatant was filtered through a 0.22-μm membrane and the protein concentration determined (Lowry et al., 1951). Different batches were done for *Nc*ESA preparation and pooled together to obtain the required protein concentration. Parasite breakdown during *Nc*ESA preparations was monitored by expression of p97, a cytosolic protein homologue of *T. gondii* that is conserved in apicomplexan parasites, as previously described (Mineo et al., 1994; Matsuura and Kasper, 1997). *Nc*ESA aliquots were stored at −20 °C until used.

2.4. Generation of BMDCs

BMDCs were generated as previously described by Lutz et al. (1999), with some modifications. Briefly, femurs and tibias were flushed with RPMI medium to release the bone marrow cells that were cultured (7×10^5 cells/well) in 24-well-culture plates in RPMI medium supplemented with 10% heat-inactivated FCS, 100 μg/ml of penicillin, 100 μg/ml of streptomycin, 5×10^{-5} M 2-mercaptoethanol (all from Sigma Aldrich, St. Louis, MO, USA), 30 ng/ml of murine granulocyte–macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml of murine recombinant IL-4 (Peprotech, Rocky Hill, NJ, USA). On days 3 and 6, supernatants were gently removed and replaced with the same volume of supplemented medium. On days 7–8, non-adherent cells were removed and analyzed by flow cytometry for DC phenotyping, given that more than 80% of the cells presented high expression of CD11c.

2.5. Chemotactic assay

Experiments were performed using a 48-well microchemotaxis Boyden chamber as previously described (Oliveira et al., 2008). BMDCs were pre-exposed to RPMI medium, lipopolysaccharide (LPS) (1 μg/ml, *Escherichia coli* 0127:B8; Difco Laboratories, Detroit, MI, USA) for 24 h at 37 °C/5% CO_2 or pertussis toxin (PTX) (1 μg/ml, List Biological Laboratories, Campbell, CA, USA) for 2 h at 37 °C/5% CO_2. After 24 h of incubation, cells were collected, washed and placed into the upper wells (50 μl cell suspension, 1.5×10^6 cells/ml in RPMI medium with 0.1% BSA (Sigma–Aldrich). Recombinant macrophage inflammatory protein 1α (MIP-1α/CCL3; 100 ng/ml, Peprotech) and 3β MIP-3β/CCL19; 500 ng/ml, Peprotech) were filled into the lower wells and served as positive controls to immature and mature DC migration, respectively. A polycarbonate filter was used to separate the two compartments (5 μm pore size; Neuroprobe, Gaithersburg, MD, USA). After incubation at 37 °C in a humidified chamber with 5% CO_2 for 1.5 h, the filter was removed, fixed, and stained with Diff-Quik (Baxter Diagnostics). The cells on the underside of the membrane were counted at 1,000× magnification (five fields for each filter). Data were expressed as the mean of the migrated cell numbers/field or migration inhibition ± SD (%). Results are representative of at least three independent experiments.

2.6. In vivo stimulation

WT and CCR5$^{-/-}$ mice were inoculated with different concentrations of *Nc*ESA via the i.p. route and sacrificed at 0, 6, 12, 24 and 48 h of stimulation. The peritoneal cavity of each mouse was washed with 2 ml of RPMI medium to determine the number of migrated cells, differential counts by Diff-Quik (Baxter Diagnostics, Düdingen, Switzerland) staining, and cell influx phenotype by flow cytometry. In some experiments, *N. caninum* tachyzoites were la-

beled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) following the previously described protocol for *Leishmania* spp. (Chang et al., 2007), with some modifications. Briefly, 1×10^7 tachyzoites were resuspended in sterile PBS containing CFSE (5 μM; Invitrogen) and incubated for 8 min at 37 °C. After incubation, CFSE was blocked with RPMI plus 10% FCS and parasite suspensions were centrifuged at 1,000*g* for 10 min at 4 °C. Stained tachyzoite (*N. caninum*-CFSE$^+$) concentration was estimated and set to dose of 1×10^6 tachyzoites/animal for inoculation in WT and CCR5$^{-/-}$ mice. No significant differences were observed in parasite viability after staining with CFSE. Mice were euthanized at 72 h p.i. and had their spleen and liver removed in order to evaluate short-term tissue parasitism.

2.7. Analyses of tissue parasitism

Spleens obtained from infected WT and CCR5$^{-/-}$ mice 72 h p.i. were dissociated using cell strainer (70 μm pore size; BD – Becton Dickinson, Sunnyvale, CA, USA), submitted to red blood cell lysis and analyzed by flow cytometry, where the percentage of splenic CD11b$^+$ cells infected with *N. caninum*-CFSE$^+$ was evaluated. FL-1 gating was determined by analysis of uninfected controls and samples obtained from mice infected with parasites stained with irrelevant IgG conjugated to fluorescein isothiocyanate (FITC). Each group/date contained three WT and CCR5$^{-/-}$ mice for the above-described experiments.

2.8. Epifluorescent histological analyses

Liver samples obtained from infected WT and CCR5$^{-/-}$ mice 72 h p.i. were embedded in freeze-mounting media (Sakura Finetek, Torrance, CA, USA), fixed in cold acetone, sectioned, counterstained with Evans blue 0.1%, nuclei stained with DAPI and examined by epifluorescent microscopy (Olympus) to evaluate whether CFSE$^+$ parasites had migrated during infection. Quantification of CFSE$^+$ parasites was performed using ImageJ software (NIH, Bethesda, MD, USA) in 40 microscopic fields per histological section (three sections/mouse/group/date) with 200× magnification. Each group/date contained three WT and CCR5$^{-/-}$ mice for the above-described experiments.

2.9. Cellular phenotyping

The distinct cell phenotypes analyzed in the experiments were properly determined through fluorescent staining with monoclonal antibodies directed to specific cell surface markers and conjugated to FITC or phycoerythrin (PE) (R&D Systems, Minneapolis, MN, USA; eBiosciences, San Diego, CA, USA), to characterize the proportion of monocytes (CD11b$^+$), DC (CD11c^{high+}) and CCR5 expressing cells (CCR5$^+$). Cell viability was confirmed by negative staining with Trypan blue and events were acquired per tube/animal/group/date. Data acquisition was performed using a FACScan flow cytometer (Becton Dickinson). All cells were analyzed by monocyte gating. Appropriate isotype-matched rat IgG was used as a negative control. Data analysis was carried out with FlowJo 8.7 software (Tree Star, Ashland, Oregon, USA).

2.10. Statistical analyses

Two-way analysis of variance (ANOVA) followed by Bonferroni post-tests were applied to compare results obtained in Boyden chamber and in vivo migration assays. In all measurements, differences were considered significant when $P < 0.05$. Statistical analysis of the data obtained was carried out using GraphPad Prism software (GraphPad, La Jolla, CA, USA).

3. Results

3.1. N. caninum antigens present chemotactic properties toward monocytes

To investigate the ability of *N. caninum* antigens to chemoattract DC, immature and LPS-matured BMDC were placed in a Boyden chamber and permitted to migrate toward different concentrations of *Nc*ESA or NLA. As shown in Fig. 1, both *Nc*ESA and NLA elicited a typical chemotactic response for immature and mature BMDC and all tested concentrations elicited cell migration compared with unstimulated controls ($P < 0.05$). *Nc*ESA presented a significantly higher, dose-dependent migration of immature BMDC, compared with LPS-matured BMDC (Fig. 1A). Although NLA also presented chemotactic properties, BMDC migration was lower compared with *Nc*ESA-induced migration. Additionally, a similar migration pattern was observed between immature and mature BMDC towards NLA, with significantly higher migration of immature cells ($P < 0.05$) being detected only at the lower concentration tested (1 μg/ml of NLA; Fig. 1B).

To test whether *Nc*ESA is also a chemotaxin in vivo, we inoculated mice with *Nc*ESA i.p. and evaluated migration of monocytes to the peritoneal cavity. As demonstrated in Fig. 2A, *Nc*ESA, in a dose of 1 μg, promoted acute monocyte recruitment to the initial site of inoculation at 24 h p.i., which was observed for higher

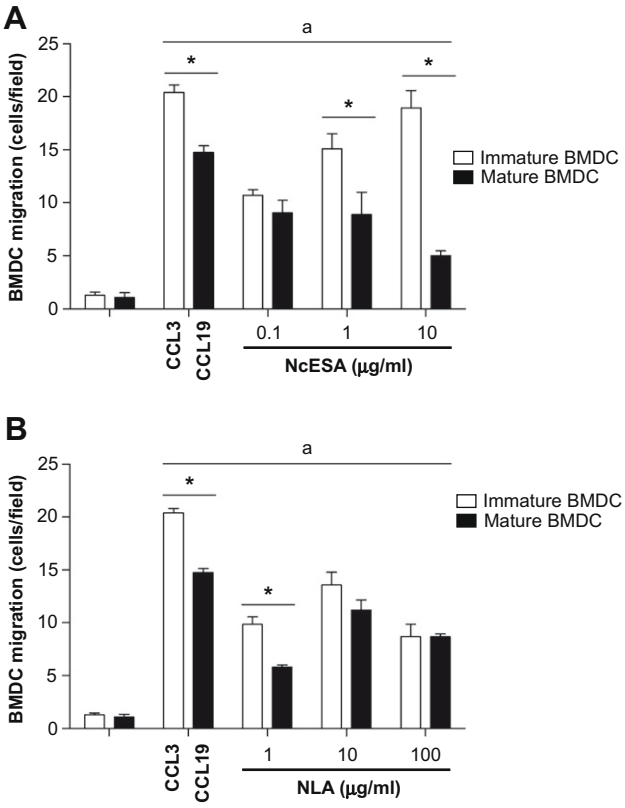

Fig. 1. Excreted/secreted antigens from *Neospora caninum* induce dendritic cell chemotaxis. Bone marrow-derived dendritic cells (BMDCs) from C57BL/6 mice were seeded in the upper wells of a Boyden migration chamber toward increasing concentrations of *N. caninum* excreted/secreted antigens (*Nc*ESAs) (A) and *N. caninum* lysate antigen (NLA) (B) in addition to recombinant chemokines CCL3 (100 ng/ml) and CCL19 (500 ng/ml). Additionally, differences in migration related to BMDC maturation were analyzed by pre-incubation of the cells with lipopolysaccharide (LPS) (Mature BMDC, ■) or left untreated (Immature BMDC, □). [a]All treatments conferred significant differences in migration compared with medium alone ($P < 0.05$). *Indicates distinct migration of BMDCs in different maturation stages ($P < 0.05$).

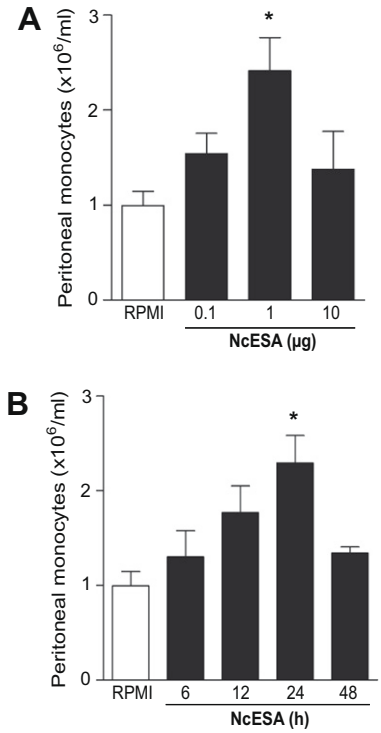

Fig. 2. *Neospora caninum* excreted/secreted antigens (*Nc*ESAs) induce in vivo monocyte migration in a dose- and time-dependent manner. (A) Increasing concentrations of *Nc*ESA (0.1, 1, 10 μg/ml) were inoculated into C57BL/6 mice ($n = 3$); (B) *Nc*ESA (1 μg/ml) was inoculated into C57BL/6 mice ($n = 3$) and time-dependent monocyte migration was assessed. After each stimulus, peritoneal exudate cells were extracted and submitted to differential counts after cytospin and Diff-Quik staining. Bars represent mean ± SD of three separate experiments. *Significant differences between peritoneal monocytes treated with *Nc*ESA and RPMI medium ($P < 0.001$).

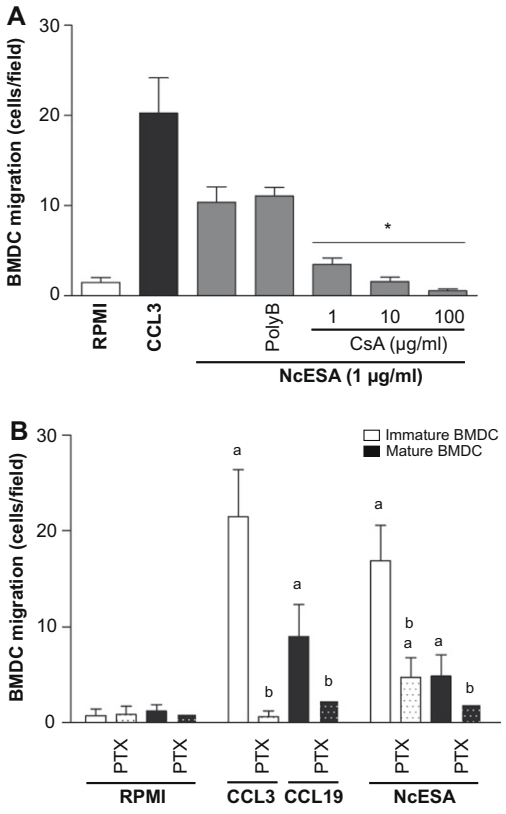

Fig. 3. Chemoattractive molecules present on *Neospora caninum* excreted/secreted antigens (*Nc*ESAs) belong to the cyclophilin family and the chemotaxis involves G_i protein signaling. Bone marrow-derived dendritic cells (BMDCs) from C57BL/6 mice were used to evaluate the effect of *Nc*ESAs on cyclophilin activity (A) or G_i protein requirement (B). (A) Immature BMDC suspension was submitted to a Boyden migration chamber using as chemotactic stimulus medium, CCL3 or *Nc*ESA (1 μg/ml), pretreated or not, with increasing concentrations of cyclosporin A (CsA; 1, 10, 100 μg/ml) or polymixin B (PolyB; 10 μg/ml). (B) Immature and lipopolysaccharide (LPS)-matured BMDC suspensions were pre-incubated with pertussis toxin (PTX; 1 μg/ml) and exposed to medium, CCL3, CCL19, or *Nc*ESA (1 μg/ml) as migration stimuli. Bars represent the percentage (mean ± SD) of BMDC migration of three independent experiments. *Significant differences between BMDCs that migrated to *Nc*ESA and *Nc*ESA pre-exposed to CsA (A, $P < 0.05$); Statistical differences in migration to the distinct chemotactic stimuli compared with medium ([a]) or induced by PTX pre-treatment ([b]) were indicated (B, $P < 0.05$).

(10 μg) or lower (0.1 μg) levels of antigenic exposure. Additionally, in vivo migration toward 1 μg of *Nc*ESA was time-dependent, with a significant increase in the peritoneal monocyte population observed at 24 h p.i. (Fig. 2B).

3.2. Chemoattractive molecules present in NcESA belong to the cyclophilin family, and the chemotaxis involves G_i protein signaling

To test whether the chemoattractant factor present on *Nc*ESA belongs to the cyclophilin family, which includes intracellular proteins implicated in a variety of cellular functions such as cell migration, we exposed *Nc*ESA to different concentrations of cyclosporine A (CsA), a major ligand of cyclophilin and a competitive inhibitor of its peptidyl prolyl isomerase activity, and observed the ability of *Nc*ESA + CsA in attracting BMDC. In these experiments, we used 1 μg of *Nc*ESA, as this concentration has previously been shown as the inducer of the greatest migration effects in vivo and in vitro. As shown in Fig. 3A, *Nc*ESA was able to attract immature BMDC in vitro; however, inhibition of parasite-derived cyclophilin by increasing concentrations of CsA significantly decreased chemoattraction of these cells induced by the secreted antigens. To eliminate the possibility of an endotoxin contaminating effect, we pretreated aliquots of *Nc*ESA with polymixin B (10 μg/ml) for 15 min before the experiment and no change was seen in BMDC migration compared with that induced by untreated *Nc*ESA (Fig. 3A). Additionally, BMDC were incubated in a Boyden migration chamber with *Nc*ESA at equal concentrations in the upper and lower well (1 μg/ml) in order to observe possible interference in the results by chemokinesis. In the absence of antigenic gradient, BMDC did not migrate to the lower chamber.

To investigate whether the chemoattraction of BMDC induced by *Nc*ESAs is involved with G_i protein signaling, migration of these cells was assessed after immature and LPS-matured BMDC were treated with PTX (toxin from the bacteria *Bordetella pertussis* that targets G_i proteins – Fig. 3B). Migration of both immature and mature BMDC to MIP-1α (CCL3) and MIP-3β (CCL19), respectively, was abolished by PTX treatment (Fig. 3B). Similarly, chemoattraction induced by *Nc*ESA (1 μg) was significantly inhibited after treatment of immature and mature BMDC with PTX (Fig. 3B). Notably, viability of BMDC was not affected by PTX treatment (data not shown).

3.3. NcESA induces monocyte chemotaxis in a CCR5-dependent manner

To evaluate whether CCR5 is involved in *Nc*ESA-induced monocyte migration, WT and $CCR5^{-/-}$ mice were inoculated i.p. with 1 μg of *Nc*ESA or medium alone. After 24 h of stimulation, the peritoneal exudate was analyzed for cell migration on the monocyte gate and we found that WT mice inoculated with *Nc*ESA presented a near threefold increase in $CD11b^{+}$ monocyte population in the peritoneal cavity compared with control WT mice. In contrast,

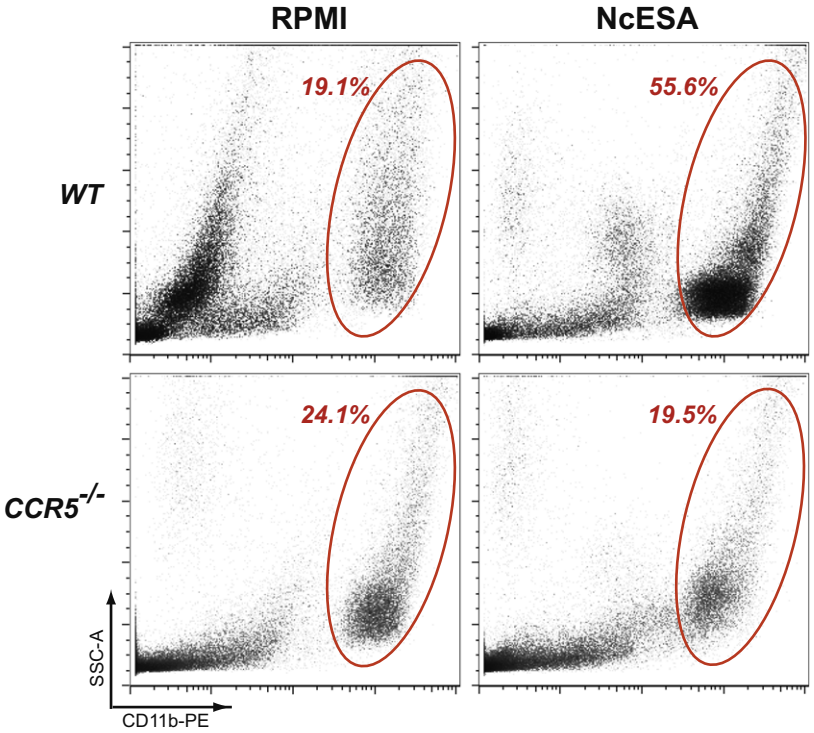

Fig. 4. *Neospora caninum* excreted/secreted antigens (*Nc*ESAs) promote acute monocyte migration to the initial site of infection in a CC-chemokine receptor 5 (CCR5)-dependent manner. Peritoneal exudate cells were extracted from wild-type (WT) C57BL/6 mice and CCR5$^{-/-}$ littermates previously inoculated i.p. with *Nc*ESA (1 μg; 24 h) or medium alone. The obtained cell suspensions were stained following described protocols for flow cytometry. Oval highlights indicate the percentage of CD11b^{+} cells inside the monocyte gate.

migration of CD11b^{+} monocytes to the peritoneal cavity of CCR5$^{-/-}$ mice was considerably abrogated after *Nc*ESA stimulation (Fig. 4). Similar results were obtained by differential cell counts performed in parallel experiments using cytospin protocols followed by Diff-Quik staining (data not shown).

To test the possibility of *Nc*ESAs also inducing CCR5 expression to ameliorate cell migration, we cultured BMDC in the presence of *Nc*ESA and evaluated the expression of CCR5 24 h after stimulation. Fluorescent microscopy showed that *Nc*ESA was able to increase the expression of CCR5 in BMDC exposed to *Nc*ESA (Fig. 5A). These data were confirmed by flow cytometric analysis, where an increment in CCR5 expression was observed after exposure to *Nc*ESA, as determined through higher mean intensity of fluorescence (MIF) and percentage of CCR5^{+} cells (Fig. 5B). In contrast, experiments performed in parallel showed that BMDC incubated with live *N. caninum* tachyzoites decreased CCR5 expression (data not shown). To evaluate *Nc*ESA-induced CCR5 over-expression in vivo, we demonstrated that after inoculum with *Nc*ESA, the peritoneal CD11b^{+}CCR5^{+} monocyte population increased over three times followed by higher expression of CCR5, compared with cells from animals inoculated with medium only (Fig. 5C).

In order to investigate whether induction of CCR5-dependent monocyte chemoattraction by *Nc*ESA constitutes an evasion mechanism used by the parasite, we hypothesized that dissemination was altered in the absence of CCR5–*Nc*ESA interaction. To test this, we evaluated parasitism on spleen and liver 3 days p.i. with *N. caninum*-CFSE and found that over 30% of splenic CD11b^{+} cells were associated with stained *N. caninum*, while only 7% of CD11b^{+} CCR5$^{-/-}$ cells were infected (Fig. 6A). Additionally, parasite spread to hepatic tissues seemed to be impaired in the absence of antigen–chemokine receptor interaction, since liver sections of CCR5$^{-/-}$ mice presented low amounts of detectable *N. caninum*-CFSE. In WT mice, labeled parasites were mostly detected inside blood vessels or at associated parenchyma (Fig. 6B).

4. Discussion

Our group recently described that MyD88, a major adaptor protein for Toll-like receptor (TLR) signaling, is critical for acute resistance to *N. caninum* infection (Mineo et al., 2009). However, inflammatory cell migration remained intact in MyD88-deficient mice, which indicated to us that chemotaxis during *N. caninum* infection is regulated by TLR-independent pathways. Based on our observations and previous work performed with *T. gondii* (Aliberti et al., 2003; Diana et al., 2005), we hypothesized that parasitic proteins could be involved in the recruitment of monocytes, a primary immune target of invasion. The data gathered in this work demonstrate that proteins, secreted during the early stages of *N. caninum* interaction with its host, recruit monocytes to the site of infection. It is well known that microbial pathogens, including viruses, bacteria and parasites, have developed many ways to evade host immune defenses (Pease and Murphy, 1998; Murphy, 2001). One tactic adopted by these pathogens is to encode homologs of chemokines and their receptors or modulate their expression (Liston and McColl, 2003). Different research groups suggest the presence of molecules on this parasite with the capacity to modulate different steps of the host immune defense (Hemphill et al., 2006).

Soluble factors from different pathogenic microorganisms have previously been shown to present chemoattractant properties per se. Bacteria and viruses have used this mechanism with success (Rao et al., 2002; Lüttichau et al., 2007). Proteins from *Staphylococcus aureus*, *Helicobacter pylori*, *Mycobacterium avium*, and *Mycobacterium smegmatis* induce chemotaxis of large numbers of monocytes, neutrophils, and eosinophils (Rot et al., 1986; Miyake et al., 1983; Castro et al., 1991; Mai et al., 1992; Rao et al., 2002). Herpesvirus, Poxvirus, together with other large DNA virus species have been shown to efficiently produce a large number of chemokine homologues, chemokine receptors and chemokine binding

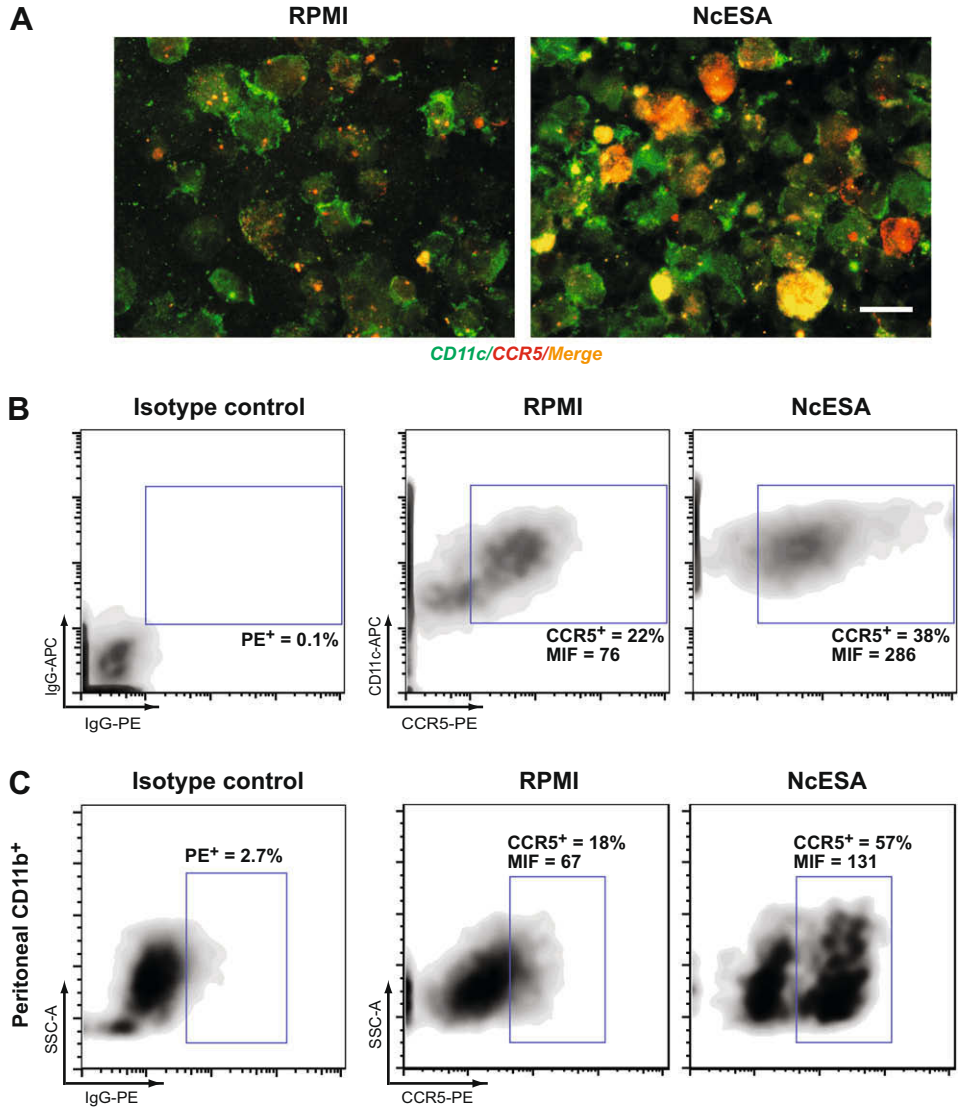

Fig. 5. *Neospora caninum* excreted/secreted antigens (*Nc*ESAs) induce over-expression of CC-chemokine receptor 5 (CCR5) in monocytes. (A) Bone marrow-derived dendritic cells (BMDCs) incubated in the presence of *Nc*ESA (1 μg/ml) or RPMI medium (control) for 24 h were observed for CCR5 expression through an epifluorescent microscope (scale bar = 10 μm). (B) Quantification of CCR5 expression was performed by flow cytometry (MIF, mean intensity of fluorescence). (C) To analyze the same effect as (B) in vivo, *Nc*ESA (1 μg/ml) was inoculated i.p. in wild-type mice (n = 3), and peritoneal CD11b$^+$ cells were measured after 24 h for positivity and intensity of CCR5 expression. PE, phycoerythrin.

proteins, with the intent of evading effector immune mechanisms through the control of host cell migration (Carfí et al., 1999; Seet et al., 2003; Paulsen et al., 2005). In our study, *Nc*ESA was able to attract monocytic cells in vitro and in vivo, verifying the capacity of the parasite to produce compounds with chemoattractive properties. *Nc*ESA was the chosen antigenic model for this work, once it required 10-fold less antigenic mass in order to induce the same chemotaxis of immature cells, compared with NLA. These results comply with previous reports and add further information in the discussion of the vital role of ESAs as major compounds in the process of cell recruitment, invasion and replication of protozoa (Denkers and Butcher, 2005; Carruthers and Tomley, 2008; Ravindran and Boothroyd, 2008). *N. caninum* is able to modulate pro-inflammatory responses in the first days of infection (Khan et al., 1997), and this recruitment in the first hours of infection may be useful to the parasite in order to invade target cells, enhancing replication. *T. gondii* has been shown to employ similar mechanisms, once the parasite is also able to control recruitment and migration of immature DC through different soluble factors (Diana et al., 2005).

Among the molecules produced and secreted by parasites with chemoattractant properties, proteins from the cyclophilin family are one of the most important candidates. Cyclophilins are conserved proteins with peptidylprolyl isomerase activity (Pratt et al., 2004) and are expressed throughout apicomplexan parasites (Krücken et al., 2009). These proteins have many intracellular functions and can be secreted (Sherry et al., 1992). Extracellular cyclophilins have demonstrated chemotactic activity in human and mouse leukocytes, including monocytes, macrophages and T-cells (Zhu et al., 2005; Damsker et al., 2007). In our model, we demonstrated that inhibition of excreted/secreted parasite-derived cyclophilins by CsA considerably abrogated *Nc*ESA chemoattraction. Similarly, it has previously been demonstrated that this parasite is able to produce and secrete proteins from the family of cyclophilins (Tuo et al., 2005). According to these authors, the described *Neospora* cyclophilin induces IFN-γ production by bovine blood mononuclear cells and antigen-specific CD4$^+$ T-cells and has its effect inhibited by CsA. Chemoattractive properties of apicomplexan cyclophilins were first demonstrated by Aliberti

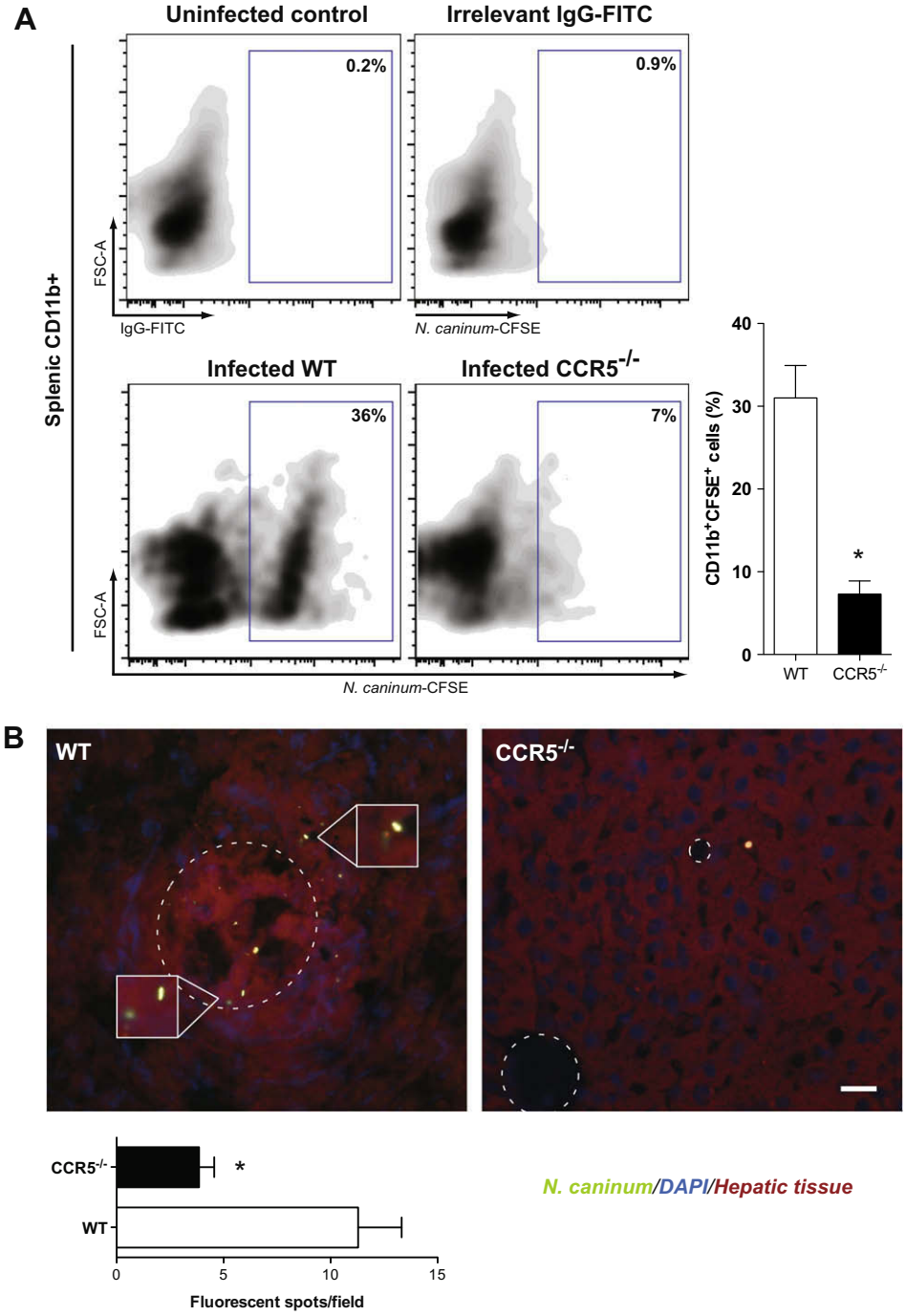

Fig. 6. In the absence of CC-chemokine receptor 5 (CCR5), *Neospora caninum* acute dissemination is impaired. *Neospora caninum* dissemination was determined by tracing carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled parasites in spleen (A) and liver (B) of wild-type (WT) C57BL/6 mice and CCR5$^{-/-}$ littermates 72 h after i.p. infection. Figures are representative of the observations performed in each group ($n = 3$ mice/group). (A) Spleen cells were gated for monocytes and CD11b^{+} cells were determined by flow cytometry. Displayed percentages indicate the number of CD11b^{+}*N. caninum*-CFSE^{+} cells. (B) Large blood vessel (circled) in a liver section of WT mice with several *N. caninum*-CFSE^{+} inside (insets; 2×), while sparse CFSE staining was observed in hepatic tissues in CCR5$^{-/-}$ mice by epifluorescent microscopy (scale bar = 50 μm). *Significant differences between CFSE detection in the analyzed tissues of WT and CCR5$^{-/-}$ mice ($P < 0.05$).

et al. (2003), who described a cyclophilin (C-18) in *T. gondii* that presents domains similar to MIP-1β, a chemokine that binds on CCR5 and recruits immature DC in vitro. A cyclophilin homologue of *T. gondii* C-18 can be found in contig 1038 of the *N. caninum* genome and this protein is most likely responsible for the migratory effects here described. However, it is not possible to discard the possibility that other proteins may be implicated in CCR5 activation and over-expression during *N. caninum* infection, since other antigenic targets described as constituents of the parasite may also bind to the receptor (Whittall et al., 2006; Monteiro et al., 2007).

CCR5 is a marked feature of immature DC and has been shown as a target receptor for C-18 (Aliberti et al., 2003), which led us to suspect that the observed monocyte migration directly involved the chemokine receptor. Chemokine receptors are a group of seven transmembrane, G_i protein-coupled receptors. Binding of G_i protein-coupled receptors by chemoattractants results in a PTX-sensitive signaling cascade that affects a variety of cell functions,

including cell migration (Fields and Casey, 1997; Le et al., 2002). Using the Boyden microchamber as a model to evaluate the effect of *Nc*ESAs on in vitro cell migration, we showed that BMDC migration to *Nc*ESA was completely inhibited by pre-treatment of the cells with PTX, showing that G_i protein-coupled receptors were involved in the chemotaxis to proteins present in *Nc*ESAs. Our results also demonstrated that the soluble factors excreted/secreted from *N. caninum* tachyzoites can control recruitment of monocytes and BMDC in a CCR5-dependent manner. CCR5 expression in monocytes is related to its immature state, and its migration to and retention at the inflammatory site (Varani et al., 2005; Vassiliou et al., 2008). Moreover, we have demonstrated that *Nc*ESA is also able to induce over-expression of CCR5 in monocytes, which seems to be very important in reinforcing the capacity of *Nc*ESAs to attract cells through the chemokine receptor. The difference in CCR5 expression between BMDC exposed to *Nc*ESA or live parasites may be explained by the maturation status of these cells. While *Nc*ESA does not appear to change BMDC maturation, the infection by live tachyzoites induces DC maturation (Teixeira et al., 2010). Antigen presenting cell (APC) maturation is marked by a sharp loss of surface CCR5 expression. In contrast, mature APC gain CCR7, the chemokine receptor responsible for APC homing to secondary lymphoid organs through a gradient of the ligand MIP-3β/CCL17 (Förster et al., 2008). It is noteworthy that CCR5 is a ubiquitous chemokine receptor and its expression in other immune cells may also be responsible for resistance against the infection or target of evasion mechanisms by the parasite. Additionally, experiments performed in our laboratory indicate that $CCR5^{-/-}$ mice were resistant to acute death after *N. caninum* infection (data not shown). In that sense, further studies are required in order to establish the complete role of CCR5 in the immune response against *N. caninum*.

As previously discussed, similar observations have been made in the *T. gondii* model. ESA from the closely related parasite were shown to modulate DC chemotaxis (Diana et al., 2005), which is most probably induced by secreted C-18 that directly binds CCR5 (Aliberti et al., 2003). Additionally, monocytes have been implied as disseminating agents of *T. gondii* to target tissues (Courret et al., 2006). These results are also in accordance with strategies of other parasites such as microsporidians, unicellular parasites which are implicated in inducing macrophage/monocyte migration in order to disseminate through host tissues (Orenstein et al., 1997). However, the CCR5/C-18 axis may not account for dissemination in *T. gondii* infection, since DC-induced parasite spread during the infectious challenge was shown to be independent of CCR5, CCR7 and MyD88 (Lambert et al., 2006). In that sense, the interaction described between *Nc*ESA and CCR5, associated to the indications provided here that the chemokine receptor may be involved in acute parasite dissemination, provides further evidence for discussion of the biological differences presented by the closely related protozoa (Innes and Mattsson, 2007).

The study presented here demonstrates that *Nc*ESA is able to chemoattract monocytic cells to the site of primary infection through the interactions between cyclophilins and CCR5. Further, we show that *Nc*ESA itself can also induce over-expression of the chemokine receptor in these cells. These mechanisms contribute to the elucidation of factors involved in migration during the early phases of infection, and may reveal evasion strategies and potential therapeutic targets to control neosporosis and its consequences.

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