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Stromal cell-derived factor-1 production by spleen cells is affected by nitric oxide in protective immunity against blood-stage *Plasmodium chabaudi* CR in C57BL/6j mice

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Abstract

Malaria, a major endemic tropical disease, is caused by the infection of blood cells by *Plasmodium* protozoa. Most patients control their parasitemia by a not fully understood spleen-dependent mechanism. SDF-1α is a chemokine produced by stromal cells such as reticular spleen cells. Nitric oxide (NO) has several immune functions, including killing of intracellular pathogens and its function in malaria is debated. We have previously shown that SDF-1α production peaks during the ascending parasitemia in *Plasmodium chabaudi* infection and its supplementation in lethal models could reduce the parasitemia. In the present study, we analyzed SDF-1 production by spleen cells as related to NO metabolism in the *P. chabaudi* rodent malaria model using IFN-γ; TNFR and iNOS-knockout mice or iNOS-blocked, L-NAME- or aminoguanidine-treated mice. Parasitemia and production of SDF-1α and SDF-1β were determined by RT-PCR. In vitro NO production by spleen adherent cells was also tested. The data showed that parasitemia was less intense in both $iNOS^{-/-}$ or NO-inhibited mice than in controls, with increased and long-lasting production of SDF-1α mRNA. In the absence of cytokines involved in the final regulation of NO production by effector cells, as is the case for $TNFR^{-/-}$ and GKO mice, the infection progressed in an uncontrolled manner regardless of SDF-1α production, suggesting that these cytokines must be involved in the control of parasitemia after the SDF-1α dependent process. The SDF-1β isoform was constitutive in all experiments, with elevated levels only clearly seen in $TNFR^{-/-}$ mice. We conclude that SDF-1 is involved in the promotion of parasitemia control in malaria, and excessive NO could affect its production.

Keywords: Nitric oxide; Protozoan parasites; Chemokine; Spleen; iNOS

1. Introduction

Malaria, a major public health problem in most tropical countries, affects 300–500 million people annually, with 0.5–2.7 million deaths attributed to the disease, especially of children in sub-Saharan Africa [1]. The disease is caused by the growth of the *Plasmodium* protozoon inside erythrocytes at a privileged intracellular site with difficult access to host immune response [2]. Most patients control the disease by destroying erythrocytes in the spleen, but also by killing parasites inside the erythrocyte. A rapid parasite clearance depends on an intact spleen, as reported for rodent malaria models [3]. This clearance is mediated both by the specific immune response and by a microenvironmental relationship between effector cells, with a highly efficient cell filtration network devoted to senescent erythrocyte removal [4].

One of the most important cells involved in the filtration network microenvironment is the reticular or stromal cell, which is functionally complex and responsible for the homing of effector cells [5]. The organization or growth of this microenvironment is regulated by several mediators, including cytokines and chemokines [6], small proteins, that are a class of inflammatory

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mediators involved in the control of homing, accumulation or mobilization of specific cells, and related to several processes associated with microenvironmental cell localization. They comprise two large classes, with more than forty molecules described associated with specific cell receptors [7]. Most of them belong to the effector and later steps of the immune response, being inducible by cytokines and produced by effector cells [8]. The second and smaller group is composed of chemokines with housekeeping function, which are produced mostly by non-inflammatory tissue cells and are devoted to organ assembling [9].

The spleen reticular cell, the cell involved in the assembling of the filtration network, produces a chemokine called stromal cell-derived factor 1 or SDF-1 [10]. This chemokine is a member of the CXCL12 or α-chemokine subfamily and is the only known ligand for the chemokine receptor CXCR4 [11]. SDF-1 and CXCR4 are constitutively expressed in a large number of tissues and also regulate embryonic development, so that knockout of those genes causes fetal loss [12]. Knockout studies have revealed that these two proteins are mandatory for various developmental processes, including chemotaxis or homing of myeloid stem cells from the fetal liver to the bone marrow environment. SDF-1 has been shown to act as a potent chemoattractant for peripheral blood cells, monocytes, pre- and pro-B cells, and CD34 human progenitors, and also blocks apoptosis of stem cells [13]. This chemokine is expressed as at least two isoforms, products of the same gene by differential splicing, SDF-1α and SDF-1β. No studies are available on their specific functions or on the molecular mechanisms that mediate these functions [14].

In malaria, effector cells act against parasites inside the erythrocyte in several ways, such as phagocytosis, but also by delivering toxic products such as free radicals and nitric oxide. Nitric oxide (NO) is a multifunctional signaling molecule that has been shown to regulate various cellular functions such as hemostasis [15], apoptosis [16], inflammation [17], vascular tone [18] and chemotaxis [19]. This free radical is produced from L-arginine by at least three isoforms of the NO synthase (NOS) enzyme: type I (neuronal NOS), type II (inducible or iNOS), and type III (endothelial or eNOS), located in diverse cytoplasmic compartments [20]. The inducible isoform of NOS (iNOS) can be expressed in murine splenic macrophages and promotes the death of several intracellular pathogens [21]. Several groups have reported a relation between protection against blood stage malaria and NO levels in serum [22]. IFN-γ knockout mice (GKO) are more susceptible to *P. chabaudi* infection and this susceptibility is related to significantly lower serum NOx (NO_2^- plus NO_3^- levels in IFN-γ Ko mice as compared with controls [23]. It was reported that TNF-α, IFN-γ and IL-12 are required in vivo to activate serum NO production during *P. chabaudi* malaria and that the enhanced NO production is related to protection [24]. Macrophages from *P. chabaudi*-infected mice produce increased amounts of NO in vitro, suggesting that this cell type is also the source of NO in vivo [25]. It was also observed that $CD4^+$ T cell clones of the Th1 type transferred protection to $CD4^+$ T cell-depleted and thymectomized recipient mice in *P. chabaudi* models, but treatment of the recipients with NOS inhibitors abrogated this protection [26]. Collectively, these findings indicate that NO production is induced during blood stage malaria in splenic macrophages by a pathway that includes IL-12, TNF-α, and IFN-γ, indicating a protective role for NO against blood stage malaria.

In contrast to these results, treatment with the iNOS inhibitor aminoguanidine (AG) does not alter the time course of *P. chabaudi* parasitemia despite the occurrence of some deaths during ascending parasitemia [27]. Another independent group confirmed these data, without any mortality and also showed that iNOS knockout mice resolve *P. chabaudi* and *P. berghei* parasitemia with the same efficiency as C57BL/6 controls, indicating that NO is not required to kill blood stage parasites [28].

These conflicting data suggest that NO production in malaria could have spectral effects according to the amounts and site of the production of this radical. For a parasite killing effect, the exact amount of NO must be delivered at the appropriate location. If an excess of microenvironmental NO induces loss of important and discrete cell populations, the absence of these cells and their products could suffice to induce severe disease by impairment of the steps where they are needed.

We recently observed that the mRNA of SDF-α is mostly produced during the early step of parasitemia control in a non-lethal rodent malaria model and when supplemented in a lethal model it induces a significant reduction of parasite burden [29]. This interaction could be ascribed to an anti-apoptotic effect of this chemokine, inducing the survival of a discrete cell population involved in the adequate immune response to malaria, which could be affected by excessive NO production, as is the case for the bone marrow dendritic cell suppression described both in human and rodent malarias [30].

In the present investigation we studied the effect of NO on SDF-1 mRNA production in the spleen during non-lethal *P. chabaudi* malaria in C57BL/6j mice by analyzing these aspects in iNOS knockout mice and by iNOS inhibition by L-NAME, and also in mice lacking crucial cytokines involved in NO production.

2. Material and methods

Reagents and specific products: all solutions were prepared with commercial reagents of at least pro-analysis quality and with sterilized 18 MΩ miIliQ®

water. When necessary, the specific origins of reagents are listed in the text.

2.1. Animals and parasites

Male WT control C57BL/6j 5–6-week-old mice were provided by our colony (Centro de Bioterismo/FMUSP) and GKO, TNFR$^{-/-}$ and iNOS$^{-/-}$ mice were provided by the colony of the Faculty of Medicine at Ribeirão Preto/USP and maintained in sterilized cages on absorbent bedding, with food and water ad libitum. The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals [31].

The CR strain of *P. chabaudi chabaudi* was a generous from Dr. D. Walliker, University of Edinburgh, UK. The parasite was stored as stabilates in liquid nitrogen between experiments to avoid any selection of virulent strains.

The malaria models were constructed using parasitized red blood cells (PRBC) from an N_2-preserved stabilate injected into a mouse of the same background. After amplification, 1×10^6 parasitized red blood cells were injected i.p. into individual mice to induce a regular experimental infection [29]. Parasitemia was monitored by microscopy in at least 1000 red blood cells of Giemsa-stained tail blood films [32]. Individual mice were deeply anesthetized and sacrificed at different time points according to each type of experimental infection, and the spleen was aseptically removed, weighed and stored for subsequent processing.

When necessary, iNOS inhibition was performed by i.p injection of 50 mg/kg/day N^G-nitro-L-arginine methyl ester (L-NAME, Sigma, St. Louis, MO) or 50 mg/kg aminoguanidine (Sigma) 2 h before malaria infection, followed by treatment maintenance with similar daily injections.

2.2. Histology

Spleen fragments were fixed by immersion in at least 20 vol. 4% formaldehyde in 0.05 M $NaPO_4$, pH 7.2, with two changes over a period of 4 h and routinely processed by paraffin embedding. Tissues sections (4 μm) were stained with hematoxylin–eosin and organ morphology was analyzed and recorded using a plan achromatic optics Leika microscope with a high-resolution digital imaging camera (Pixera).

2.3. Spleen cell culture

Spleens from normal or infected C57BL/6j mice were aseptically removed and dissociated with scissors and needles, until a uniform cell suspension was obtained in 10 ml of RPMI 1640 medium without phenol red (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (WL Imunoquimica, Brazil), 0.2% glutamine and 10 μg/ml gentamicin. The cells were recovered by centrifugation at 1000 rpm for 10 mm and erythrocytes were removed by adding 1 vol. of cold 0.17 M NH_4CL, pH 7.4, with gentle shaking, followed by filtration of the cell suspension through sterile gauze. After recovery, cells were washed twice with phosphate buffered saline (PBS), pH 7.2, by centrifugation, suspended in the same media and counted. Cell viability was determined by Trypan blue exclusion, and preparations showing less than 90% nucleated cell viability were discarded. Adherent spleen cell cultures were established by seeding 100 μl of a splenocyte suspension produced as described above and suspended in complete medium at a viable cell concentration of 5×10^6 cells/ml on a 96-well tissue culture plate. The plate was incubated at 37 °C in a moist 5% CO_2 atmosphere for 1 h for cell adherence. After manual shaking, the medium was removed and the plate refilled with complete medium. The process was repeated twice for complete non-adherent cell removal.

Specific stimulation of adherent cells was performed by adding purified *Escherichia coil* LPS (Sigma) to a final concentration of 200 μg/ml in cell medium, with non-stimulated wells as controls.

2.4. NO determination

NO production was detected in spleen adherent cells obtained sequentially after malaria infection and produced as described above. The cells were allowed to adhere for 1 h and incubated for 48 h in 5% CO_2 in complete medium with or without *E. coil* LPS. Supernatants were collected and assayed for NO on the basis of total nitrogen ions (NOx) determined with an ozone chemiluminescence system after vanadium catalysis (Sievers® nitric oxide analyzer) [33]. In addition, nitrite concentration was calculated by the Griess reaction using $NaNO_2$ as a standard, without any catalytic or enzymatic NO_3–NO_2 conversion [34].

2.5. Nucleic acid extraction and purification

Chemokine mRNA expression was determined by applying semi-quantitative RT-PCR to RNA obtained from a pool of spleens from three animals at each experimental time. The spleens were weighed and immediately dissociated mechanically with fine tip scissors in 4 vol. (w/v) of Trizol® for RNA extraction. The cell suspension was mixed in a vortex mixer and stored at −70 °C for further processing. RNA extraction was performed by adding chloroform (0.2 vol.) to the Trizol suspension, vortexing and centrifuging at 11 000 g/15 mm to recover the aqueous phase. The RNA was precipitated by adding isopropyl alcohol (1:1), with incubation for 15 min at −20 °C. After centrifugation, the RNA pellet was washed with one volume of 75%

ethanol in water. The total amount of nucleic acid and its purity were determined by spectrophotometry, and low purity batches were re-extracted. The nucleic acid concentration of the final preparation was determined spectrophotometrically in water and each sample was adjusted to a final concentration of 0.5 μg RNA/μl, aliquoted and stored at −70 °C until the time for use.

2.6. Reverse transcriptase and polymerase chain reaction (RT-PCR) for the detection of chemokine mRNA

Complementary DNA (cDNA) was synthesized from 5 μm of total spleen RNA using oligo (dT) primers (0.5 μg/reaction) and reverse transcriptase (Super Script™ II RT-Gibco, 200 U/reaction), in 50 mM Tris–HCI, 75 mM KCl, 3 mM $MgCl_2$ and 0.01 DTT, containing 1 mM of dNTPs in a final volume of 25 μl. The reaction was incubated at 37 °C for 1 h and immediately used or stored at −70 °C. PCR was conducted in a programmable thermal cycler PTC-100 (MJ Research Inc.) using 5 μl of cDNA/reaction, 10 pmol of specific primers, with Taq DNA polymerase (GibcoBRL, 1 U/reaction) in 20 mM Tris–HCI, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and 0.2 mM dNTPs. The reaction was conducted for 28 cycles (denaturation, 1 min, 94 °C; annealing, 1 min, 54 °C; amplification, 2 min, 72 °C). The primer sequences, elsewhere described [21], were: SDF-1α (product size 348 bp): sense CTCCAAACTGTGCCCTTCAG, antisense AAAGCTCCATTGTGCACGGG, SDF-1β (product size 368 bp): sense CCGGAATTCCTCCAAACTGTGCCCTTCAG, antisense CCGGAATTCGCCTGTCACCAATGACGTTG; β-actin [product size 349 bp): sense TGGAATCCTGTCGCATCCATGAAAC, antisense TAAAACGCAGCACAGTAACAGTCCG. The PCR products were separated by usual 6% polyacrylamide gel electrophoresis with silver staining [35], using consecutive lanes for β-actin, SDF-1α and SDF-1β for each experimental time and animal. Occasionally, specific bands were quantified on dried gels using the ImageJ free software (http://rsb.info.nih.gov/ij/). Values were expressed as percent β-actin mRNA after background subtraction, using the stained gel bands of β-actin RT-PCR product in the same reaction as standard unit.

2.7. Statistical analysis

Data were analyzed statistically by ANOVA followed by the Bonferroni test to determine subgroup differences using the Graph Pad Prism 3.0 package, with the level of significance set at $p < 0.05$.

3. Results

3.1. Spleen cell culture and NO production

We studied NO production by adherent spleen cells from at least three *P. chabaudi-infected* mice per experimental time. The NO and NOx produced by these cells in culture medium 48 h after seeding with or without LPS stimulation, as related to parasitemia evolution, is presented in Fig. 1. The figure shows that the highest NO production occurred before the parasitemia peaks, between 3 and 6 days after infection, decaying thereafter to basal levels. This early production by adherent cells presented a marked LPS-dependent NO increase which was much lower after parasitemia control. This pattern was observed when colorimetric nitrite determination was performed in the same experiment as an additional detection system.

3.2. Parasitemia and SDF-1α production in mice treated with NO inhibitors

We infected several groups of C57BL/6j mice with 10^6 parasitized erythrocytes as described in Section 2. Parasitemia was monitored in tail blood and groups of animals were killed during the course of non-lethal disease. Spleen removal, RNA purification and SDF-1α and β mRNA determination followed, as illustrated in Fig. 2A. In agreement with the data obtained for NO determination, parasitemia reached 40% levels by the 8th and 9th day after infection, decaying thereafter and practically disappearing on the 14th day.

SDF-1 mRNA production was different for each isoform, being almost constant for SDF-1β, unrelated to parasitemia, but with a clear SDF-1α mRNA production occurring at early time points, i.e. 4 and 6 days after infections, with a new re-appearance only during convalescence.

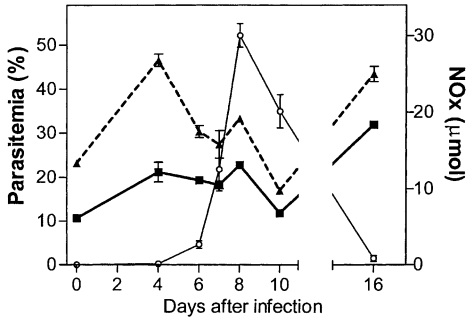

Fig. 1. Production of NO free radicals by splenic adherent cells during *P. chabaudi* infection in C57BL/6j mice. Total NOx (nitrate and nitrite) production in 48 h culture medium. Symbols: medium from non-stimulated cells is represented by a solid line and squares, medium from LPS-stimulated cells as a dashed line and solid triangles, as compared to the parasitemia observed at spleen collection (circles). Bars represent the S.E.M.

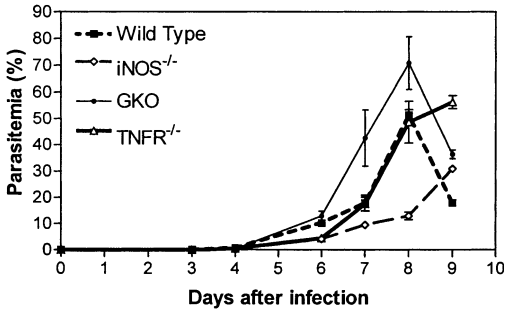

Fig. 2. Evolution of malarial infection in C57BL/6j mice and in genetic knockout mice. Wild type mice are represented by squares, iNOS$^{-/-}$ by diamonds, GKO by circles and TNFR$^{-/-}$ by triangles. Errors bars represent the S.E.M.

Similar groups of mice were treated daily i.p. with 50 mg/kg L-NAME (185 μM), a full NOS inhibitor, or aminoguanidine (367 μM), an iNOS selective inhibitor at this concentration. The treatment was started 2 h before malaria infection and was maintained throughout the experiment, with non-infected treated animals being used as controls. There was a clear reduction in parasitemia in treated animals, with a smaller and shorter peak ($p < 0.05$) compared to controls (Fig. 2A) reaching a maximum by 8–10 days, followed by subsequent parasitemia control regardless of whether L-NAME (Fig. 2B) or aminoguanidine (Fig. 2C) was used as inhibitor. SDF-1 mRNA production in the spleen of the three groups according to parasitemia evolution and was practically abolished at most experimental times in L-NAME-treated animals. This was especially true for the β isoform, as compared to control animals, whose SDF-1α mRNA expression presented a clear increase at early times, disappearing during the parasitemia peak but occurring again after parasite clearance. In aminoguanidine-treated animals, SDF-1 mRNA expression of both isoforms was higher compared to control and was maintained throughout the infection.

3.3. Evolution of P. chabaudi infection in knockout mice

Similarly to the experimental model described above, we infected and followed groups of mice with knockout genes in terms of NO production or induction. Groups of GKO, TNFR$^{-/-}$ and iNOS$^{-/-}$ mice were also monitored for parasitemia, as illustrated in Fig. 3. GKO mice presented severe infection with higher parasitemia ($p < 0.05$) at early times and reached a higher parasitemia peak (80%). Despite the severity of their condition, none of these mice died, similar to the control at this experimental time. TNFR$^{-/-}$ mice presented parasitemia closely similar to that of wild type animals, with similar peak levels on the 9th day of infection followed by sustained progression of the infection, without the decay and control observed in wild type animals. The parasitemia of iNOS$^{-/-}$ mice was lower and of short duration as observed in wild type infected mice, and similar to that observed in L-NAME-treated mice. The levels of parasitemia were lower, reaching 30% only at later experimental times.

3.4. Expression of SDF-1α mRNA in knockout mice during P. chabaudi infection

Groups of knockout mice and their wild type controls were infected as described above described and killed according to disease progression, with spleen removal. After RNA extraction, RT-PCR for SDF-1 mRNA was performed and quantified as described in Section 2, and the results are illustrated in Fig. 4, together with descriptive band scans. WT animals presented SDF-1 production similar to that described above, with early production of SDF-1α mRNA and sustained SDF-1β production despite faint bands (Fig. 4A). iNOS$^{-/-}$ mice presented more constant SDF-1α mRNA production during infection, with higher production at the last experimental time. SDF-1β presented the same pattern as described for L-NAME treated animals, with disappearance of this mRNA at the peak of infection, followed by an upsurge at the last experimental time, when parasitemia control began. TNFR$^{-/-}$ mice had higher levels of SDF-1α mRNA before infection, followed by a decay and an upsurge at the last experimental time points. The expression of the SDF-1 β isoform was low in non-infected mice but increased during infection. GKO mice presented a higher level of SDF-1α and SDF-1β production before infection, as the absence of IFN-γ result in higher basal levels of SDF-1. When basal levels were compared to those observed during infection, SDF-1α levels decayed during the parasitemia peak to upsurge thereafter, in agreement with results obtained with other models. The production of SDF-1 β mRNA was more stable and constant during malaria in these GKO mice.

3.5. Spleen histology in mice with interference in NO production

We analyzed the spleen morphology of mice infected with *P. chabaudi,* whose NO production was affected by NOS inhibitor treatment, and of genetically modified iNOS mice. We examined both non-infected animals and control non-infected genetically modified mice, with same iNOS inhibitors treatment, as illustrated in Fig. 5. The red pulp showed less intense phagocytosis and fewer pigment-loaded macrophages in both models, which also presented lower parasitemia compared to control. In the white pulp there was an apparent packing of lymphoid cells in follicles in L-NAME-treated animals or iNOS$^{-/-}$ animals compared to controls, but without a clear morphological distinction (data not shown).

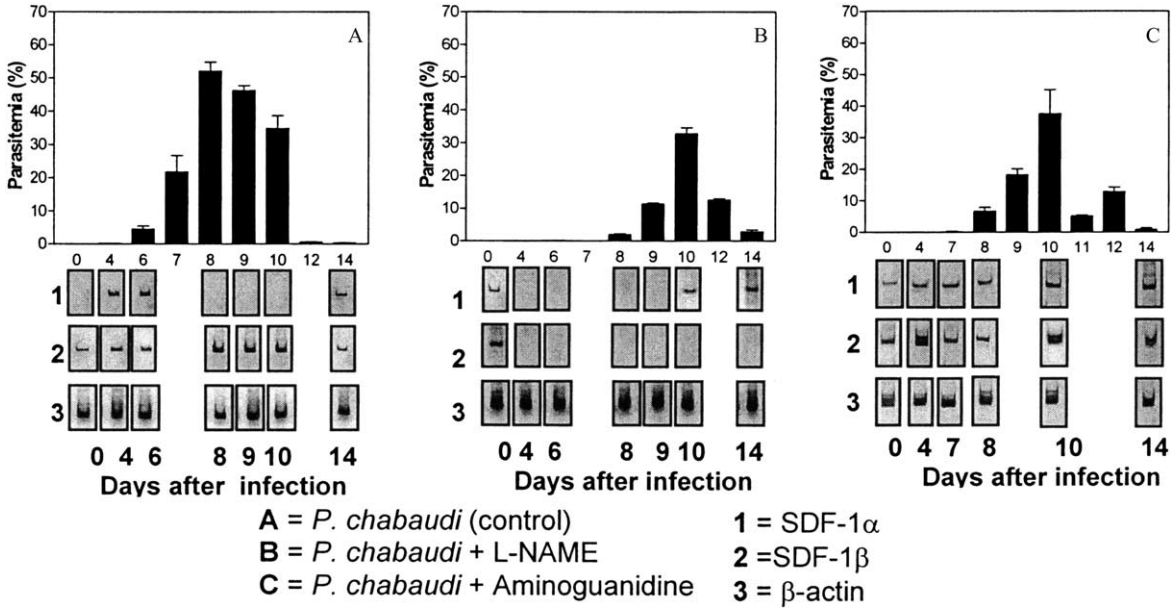

Fig. 3. SDF-1 expression in the spleen during *P. chabaudi* malaria in mice treated with NOS inhibitors, as detected by RT-PCR. (A) Control mice. (B) L-NAME (185 μM) and (C) aminoguanidine (367 μM). The upper chart presents the evolution of parasitemia, with error bars representing the S.E.M. The lower part presents digital images of specific mRNA products. (1) SDF-1α; (2) SDF-1β; (3) β-actin.

3.6. Spleen histology of cytokine knockout mice

We performed the histological analysis of the spleen from knockout animals, infected or not with *P. chabaudi*, collected at the peak of infection. The white pulp showed irregular cellularity in GKO animals, with monocytic cell infiltration, while TNFR$^{-/-}$ mice presented a compact non-activated cellular pattern. In the red pulp, GKO animals presented lower phagocytosis and pigmentation compared to control despite their higher parasitemia, while TNFR$^{-/-}$ mice presented a less evident reduction of phagocytic activity (Fig. 6).

4. Discussion

We demonstrated a clear inverse relationship between iNOS-produced NO and SDF-1 mRNA production. The sequential production of NO-related radicals by spleen adherent cells was higher before and after intense parasitemia, being normal or near normal during the parasitemia peak. Although these data are similar to previously reported results [36], this production was quite low, as also observed in *Leishmania*-infected macrophages [37]. The importance of this radical for the spleen has been suggested by other authors by mRNA iNOS detection [27] using the same model, but without a clear direct relationship between NO production and direct parasite killing. The increased NO production detected here in adherent cells obtained from the early low parasitemic stages of infection could be ascribed to low specificity IL-12-dependent IFN-γ production by NK cells observed both in *P. chabaudi* models at the same time points [38] but also in in vitro *Plasmodium*-activated PBMC from non-infected humans [39]. This could explain the higher LPS activation observed in our study at these time points, in contrast to the low LPS activation observed during convalescence, due to the production by specific cells which is less LPS dependent The absence of a clear increase on NO production by adherent cells in intense parasitemia may represent indirect evidence that this radical is probably not involved in direct parasite killing. The killing of parasite-infected erythrocytes probably occurs after phagocytosis by activated macrophages using more classic radicals such as oxygen or halogen radicals [40], since oxygen radical production was found to be most important in parasitemia control in several malaria models [41].

The present study confirms the role of IFN-γ in the protective immune response to blood-stage malaria, as described elsewhere for human disease [42] and using our malaria model [23]. Using GKO mice infected with the CR strain of *P. chabaudi*, we observed high parasitemia levels compared to wild-type mice. Despite a similar effect in human disease [42], TNFR knockout mice developed parasitemia similar to WT mice during the ascending phase of the acute infection, suggesting a secondary role of this chemokine in parasitemia control during this step of infection, as described by others [36]. Interestingly, iNOS knockout mice presented lower parasitemia as compared to WT, as also suggested by others in similar malaria models [43]. This, however, contrasted with other infections with intracellular

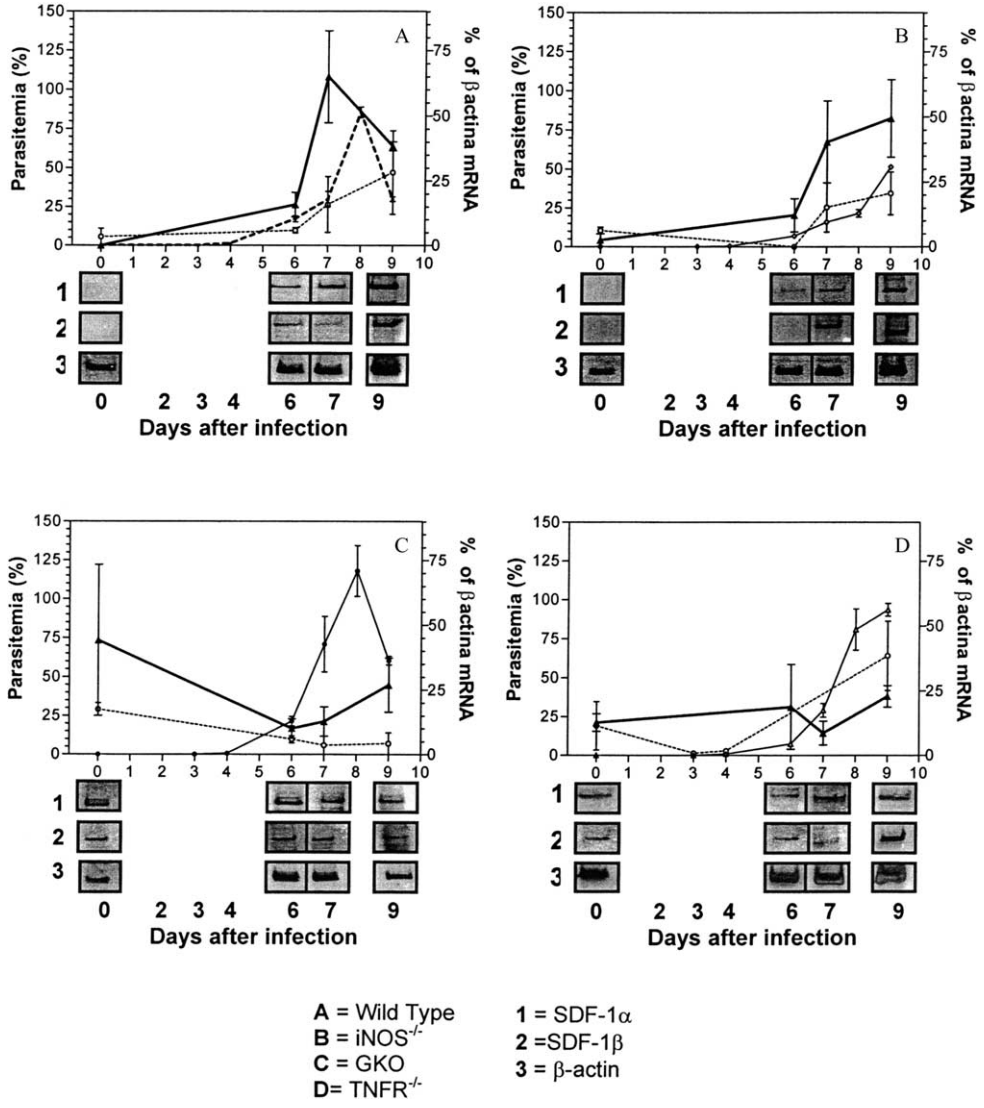

Fig. 4. SDF-1 mRNA expression on the spleen during *P. chabaudi* malaria in knockout mice for enzymes or cytokines involved in NO production, as detected by RTPCR. (A) WT; (B) iNOS$^{-/-}$; (C) GKO; (D) TNFR$^{-/-}$. Parasitemia is expressed as a dashed line with squares, quantified SDF-1α mRNA as a solid line and triangles, and quantified SDF-1β mRNA as a dashed line with open circles. Error bars represent the S.E.M. for group measures. The lower part of each chart shows representative digital images of specific mRNA products. (1) SDF-1α; (2) SDF-1β; (3) β-actin.

pathogens such as *Trypanosoma cruzi,* when iNOS$^{-/-}$ mice presented higher parasitemia and severe disease [44]. As suggested by others [45], the explanation for this finding is a regulatory effect of NO early during infection, as also shown by us, with induction of apoptosis of a specific group of cells. This compromises the ability of the host to achieve a rapid immune response, which was abolished in iNOS$^{-/-}$ mice, with an earlier and effective control of parasitemia.

We had shown previously that SDF-1α is produced during the early stages of parasitemia in this model, and its supplementation in lethal models could induce a better immune response to infection [29]. In the present study we investigated the production of SDF-1 mRNA by RT-PCR using models with inhibition of iNOS or both eNOs and iNOS. Controls presented the typical pattern of early production of the SDF-1α isoform of this chemokine, while the β isoform was produced in a constitutive manner. Parasitemia was lower both in mice tread with L-NAME, a non-selective NOS inhibitor, and in mice treated with aminoguanidine, an iNOS inhibitor [46], with related spleen histology, but with diverse effects on SDF-1 mRNA production. iNOS inhibition clearly induced the mRNA of both isoforms in the spleen at most of the experimental time points, while complete NOS blockade appeared to affect the production of both isoforms. Thus, iNOS-inducible NO production appears to play a regulatory effect, blocking the production of SDF-1α mRNA, while the complete blockade of NO production could interfere with another level of SDF-1 production, probably in endothelial cells, affecting the production mostly of the β isoform of

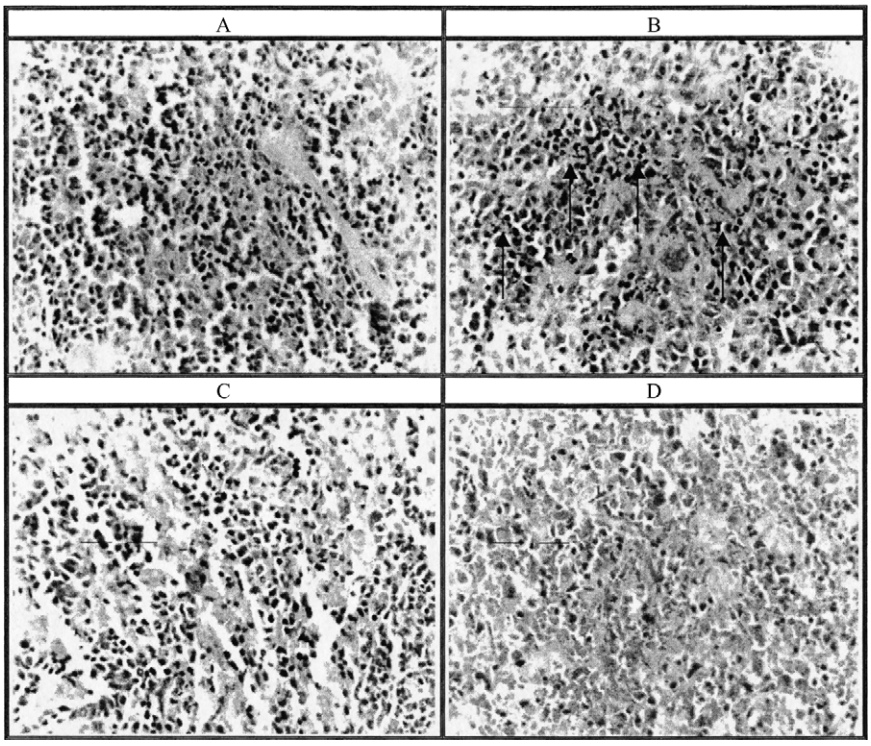

Fig. 5. Histological pictures of spleen red pulp from mice with chemically or genetically abolished NO production during *P. chabaudi* malaria. The figure shows representative digital fields of HE-stained red pulp areas observed with a 10 × planachromatic objective. (A) WT non-infected mice; (B) WT infected with *P. chabaudi*; (C) L-NAME-treated mice infected with *P. chabaudi*; (D) iNOS$^{-/-}$ infected with *P. chabaudi*? Infected animals were killed on the 9th day of infection, with L-NAME-treated or iNOS$^{-/-}$ mice presenting lower phagocytosis and less pigment (arrows in controls) in both models, according to their lower parasitemia.

SDF-1, but not affecting the early steps of the immune response which could control the parasitemia, since we found better control of parasitemia in both models together with similar low intensity phagocytosis in red pulp at the height of the infection. iNOS knockout animals showed the same profile as that of aminoguanidine-treated animals, with higher SDF-1α mRNA production, without affecting the β isoform. In GKO and TNFR$^{-/-}$ mice, a similar effect with high SDF-1 mRNA production was found, but resulting in similar or higher parasitemia. This chemokine was present at higher levels in the spleens of non-infected GKO mice, with lower but still high levels in non-infected TNFR$^{-/-}$ mice. After infection, GKO mice presented an increase of this mRNA in the spleen, without parasitemia control. At histology, the spleen found to contain large numbers of inactive monocytic cells in the white pulp and low numbers of pigment- laden macrophages, as compared to controls, probably due to a constant recruitment of cells, that was also found in non-infected animals and described elsewhere [47]. These cells fail to became activated due to the absence of IFN-γ, without iNOS NO production and without their regulatory effect, as proposed by others [48]. These data support the idea that SDF-1 must be involved in promoting homing of effector cells that, in the absence of effective activation, become resident but inactive cells, without parasite clearance.

Similar data were obtained with TNFR$^{-/-}$ mice, without the same intensity, probably due to a less important pro-inflammatory activity of this cytokine in this malaria model, as suggested by others. An interesting fact observed was the extremely high presence of the SDF-1β isoform mRNA in the spleen of infected TNFR$^{-/-}$ mice, suggesting that some inhibitory effect of this cytokine could affect the production of this isoform in WT animals. This fact may be related to blood forming cells since TNF has a diserythropoietic effect related to malarial anemia, as described elsewhere [49], indicating that this isoform is associated with recruitment of blood forming cells.

Few studies were performed with SDF-1 and malaria, but thus chemokine has been suggested to have an anti-apoptotic effect [13] in bone marrow transplantation and is frequently found in chemokine cocktails for stem-cell rescue [50]. Here we suggested that in our rodent malaria model the SDF-1-producing cells must be down-regulated by NO produced by iNOS, depending on IFN-γ and TNF-α pro-inflammatory activation. NO production by spleen adherent cells during early infection in fact favors the upsurge of parasitemia and not its control. These data contrast with those obtained for classic intracellular parasites [51] for which the produc-

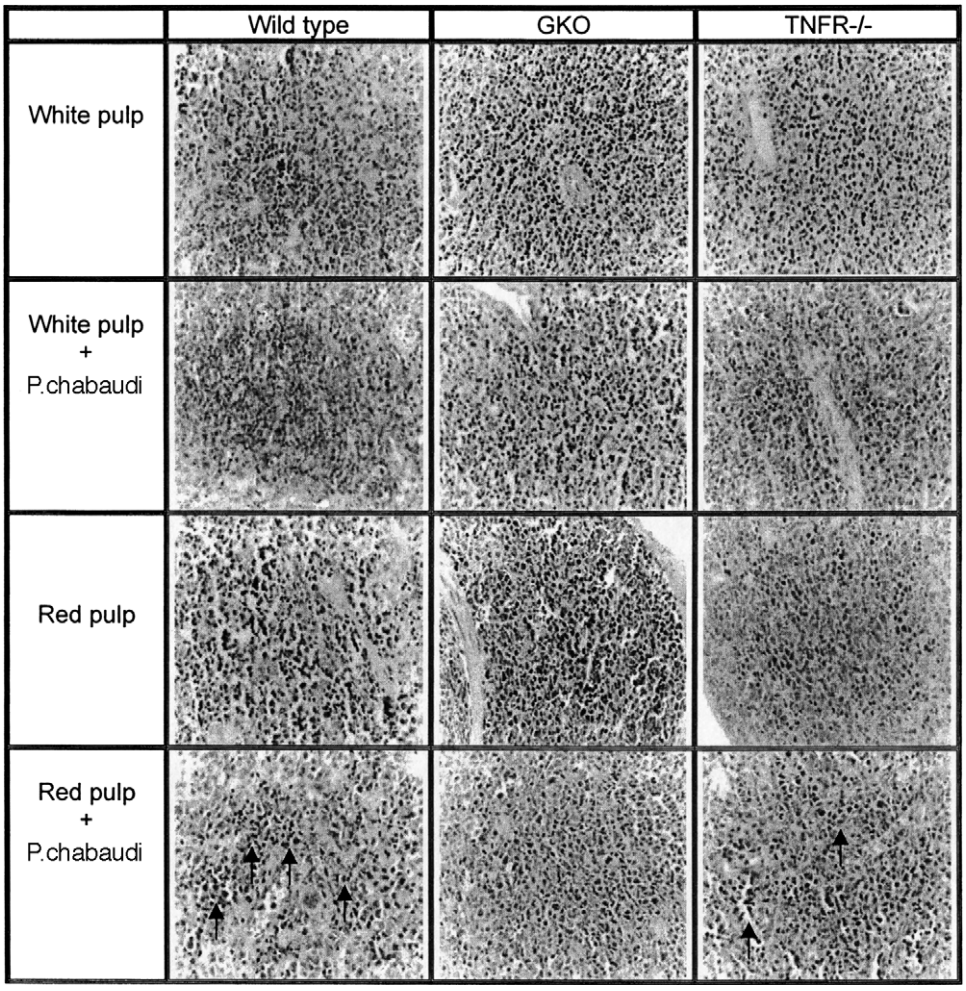

Fig. 6. Histological pictures of spleens from cytokine knockout mice infected with *P. chabaudi* and adequate controls. The figure shows representative digital fields of HE stained white and red pulp areas observed with a 10 × planachromatic objective. Infected animals were killed on the 9th day of infection. White pulp shows irregular cellularity in GKO animals, with monocytic cell infiltration, while $TNFR^{-/-}$ mice present a compact nonactivated cellular pattern. In the red pulp, GKO animals present lower phagocytosis and pigment as compared to controls despite their higher parasitemia, while $TNFR^{-/-}$ mice present less evident reduction of phagocytic activity (arrows).

tion of NO is considered microbicidal and essential for disease control.

Despite their intracellular location, the erythrocytic malaria parasites are not classic intracellular ones because they always destroy the infected host cell within a few days, while most of their counterparts are chronic intracellular inhabitants that must maintains their host cell living for their survival. Changes in host cell metabolism by inflammatory or immune factors such as IFN-γ or NO are not expected in our model due to the infected cell type, the erythrocyte, but also due to the extremely rapid cell involvement and destruction. In fact, the response of the host is fully devoted to the killing of both the infected cell and the parasite, which could be considered a unit of an extracellular parasite, and this killing process must depend on the growth and construction of effector microenvironments, with an important participation of SDF-1. Our data indicate that NO free radicals produced by iNOS could interfere with this SDF-1 production and affect the early steps of the adequate immune response in non-lethal *P. chabaudi* rodent malaria.

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