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Stromal cell derived factor 1 synthesis by spleen cells in rodent malaria, and the effects of in vivo supplementation of SDF-1α and CXCR4 receptor blocker

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Abstract

The mechanisms of malaria parasite clearance in the host are not well understood, but are ascribed to the intact spleen, the site for parasite clearance. The infection induces a huge increase in spleen volume and cellularity. There is, however, a lack of studies on the splenic production of chemokines, which are small proteins that control homing and activation of immune cells and must be crucial for organized tissue growth. We studied the spleen cell production of SDF-1, a primordial chemokine of the CXCL12 class, through mRNA Reverse transcriptase and polymerase chain reaction of both isoforms, α and β, in lethal (*Plasmodium berghei* ANKA) and non-lethal recrudescent malaria (*Plasmodium chabaudi* CR) in BALB/c and C57BL/6 mouse strains. In non-lethal *P. chabaudi* malaria in C57BL/6 mice, SDF-1α mRNA production clearly peaked before the control of parasitemia, a fact not observed in the same mouse strain infected with lethal *P. berghei*, when this production was lower and without peaks. The infection of BALB/c mice infected with the same *Plasmodium* species led to a similar evolution of parasitemia and also chemokine production, albeit at lower levels. SDF-1β synthesis was more constant and regular during both infections, presenting some variation but usually occurring at all the experimental times. Supplementation of lethal models with SDF-1α i.p., at the time when endogenous stromal cell chemokine production peaked in non-lethal models, induced a clear reduction in parasitemia, probably with prolonged host survival. Blocking SDF-1 action by administration of T-140, a CXCR4 receptor blocker, caused an increase in circulating parasites in the usually benign non-lethal *P. chabaudi* malaria in C57BL/6 mice, mainly at recrudescence of parasitemia. These data suggest that SDF-1α production in the spleen plays an important role in rodent malaria, and its supplementation was found to partially correct defects in the control of malaria in lethal models.

Keywords: Protozoan parasites; Chemokines; Spleen; Rodent; Stromal cells

1. Introduction

Malaria, a most important human parasitic disease, is caused by protozoans of the genus *Plasmodium*, which infect erythrocytes. Each year, 2–3 million children die as a result of *Plasmodium falciparum* malaria, and up to 500 million people throughout the world present with the clinical disease [1]. Despite extensive research, some aspects of this infection remain obscure, such as the effector mechanisms for the control of parasitemia, which must be quick and potent, in view of the observed early elevation of circulating parasites and their rapid clearance. The spleen has been associated with this process in humans and also in rodent malaria models; parasite clearance appears to be dependent on the splenic filtration network that is usually devoted to senescent erythrocyte clearance and is controlled by reticular cells [2].

Recently, a novel and large class of inflammatory mediators, the chemokines, has been described. These

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molecules are involved in the attraction and accumulation of leukocyte subsets, especially discrete leukocyte populations [3], in acute and chronic inflammatory processes in several diseases [4]. A new dichotomous classification based on function proposes two large groups, the inflammatory and the homeostatic chemokines [5], but this distinction is not absolute; many features are ascribed to both groups, with definite profiling requiring further research [6].

One of the most important splenic functions has been associated with the clearance of not only senescent erythrocytes but also intraerythrocytic parasites, such as *Plasmodium* [7]. The functionally complex stromal or reticular cells control this process, producing various chemokines and cytokines, but their unique specific products are the stromal cell derived factors 1 (SDF-1), related to homing and activation of precursor cells [8] and recognized only by the CXCR4 receptors [3]. These receptors are found mainly in CD34+ cells, as macrophages, haematopoietic precursors, T cells, monocytes, and haematopoietic stems cells both in mice [4] and humans [9,10]. The three observed isoforms, SDF-1α, SDF-1β and SDF-1γ, probably result from differential splicing of a single gene, with SDF-1α lacking four amino acids in its carboxy-terminal end [8]. SDF-1 regulates several tissue functions, both in organogenesis [11] and in inflammation [12], which depend on both the prevalence of the CXCR4 receptor and on other mediators, like nitric oxide [13]. The binding of SDF-1 to its receptor is blocked by T-140, a truncated polyphemusin peptide analogue that was shown to efficiently inhibit infection of target cells by T-cell line-tropic strains of HIV-1 through its specific binding to CXCR4 [14].

Rodent malaria is a useful experimental approach and, despite some constraints, the choice of the parasite and recipient mouse strains creates animal models that resemble each type of human disease [15]. *P. chabaudi* CR strain represents self-controlling experimental malaria, appropriate for studies on blood stage immunity [16]. Some other strains of *P. chabaudi* cause lethal malaria, with death ascribed to intense parasitemia (> 80%) and anemia but without the usual aspects seen in man [17]. *P. berghei*, on the other hand, represents lethal mouse malaria, with brain involvement in specific mouse strains such as CBA or C57Bl/6j and showing several features that resemble the human disease [18]. The opposite outcomes of these experimental malarias permit the comparison of acute splenic responses [19]. The spleen has been implicated as the main site of *Plasmodium* clearance, apparently due to the rapid and synchronous growth of the filtration network controlled by reticular cells; their subsequent activation could explain the rapid clearance of parasitized erythrocytes [7]. Reticular cells belong to the group of stromal, reticular or dendritic cells, which produce specific chemokines such as SDF-1 [4]. Their function during malarial infection may be evaluated by measuring the synthesis of splenic SDF-1 mRNAs.

In this work, we studied the splenic mRNA expression of selected chemokines, SDF-1α and -1β, during sequential experimental infection of BALB/c and C57Bl/6j mice with *P. chabaudi* and *P. berghei*. We also tested the effect of administering synthetic or recombinant SDF-1α to mice during lethal *P. berghei* malaria or of blocking endogenous SDF-1 action by T-140 supplementation during infection with non-lethal *P. chabaudi*.

2. Methods

2.1. Animals, parasites and specific reagents

Male, inbred 5–6-week-old BALB/c or C57BL/6 mice were used in the experiments and were provided by our colony (Centro de Bioterismo/FMUSP). The animals were maintained in sterilized cages and absorbent media, with food and water ad libitum, and were handled in accordance with the Guide for the Care and Use of Laboratory Animals [20]. Parasite strains, *P. chabaudi* CR strain and *P. berghei* ANKA, were the generous gift of D. Walliker, University of Edinburgh, UK; they were stored as stabilates in liquid nitrogen between experiments, to avoid any selection of virulent strains. Human recombinant SDF-1α was a gift of PeproTech Inc., Rocky Hill, USA. Mouse synthetic SDF-1α and T-140 were kindly provided by N. Fujii, Kyoto University of Kyoto, JP.

2.2. Experimental malaria models

Parasitized red blood cells (pRBC) from a liquid N_2 preserved stabilate were injected in a mouse of the same background. After amplification, 1×10^6 pRBC were injected intraperitoneally in individual mice to induce a regular experimental infection. Parasitemia, expressed as percent of infected erythrocytes, was monitored by Giemsa stained tail blood films, and determined in at least 1000 red blood cells by microscopic examination, by two independent observers [21]. Mice were killed at experimental times by carbon dioxide inhalation; the spleens were aseptically removed and immediately processed.

2.3. Nucleic acid extraction and purification

Spleen chemokine mRNA expression was determined by semi-quantitative RT-PCR. In a typical experiment, whole spleens were aseptically removed from groups of three animals per experimental time, weighed, pooled and immediately and mechanically dissociated with fine

tip scissors in four volumes (w/v) of TRIZOL®. The cell suspension was vortexed and stored at −70 °C until further processing. RNA extraction was performed by addition of chloroform (0.2 volume), vortexing and centrifugation at 11 000 × *g*/15 min, with recovery of the upper aqueous layer. RNA was precipitated by adding isopropyl alcohol (1:1), followed by incubation for 15 min at −20 °C and recovery by centrifugation; the RNA pellet was then washed with 1 volume of 75% ethanol in water. The total amount of nucleic acid and its purity was checked by spectrophotometry, and low purity extractions were reprocessed. Each sample was adjusted to 0.5 mg of RNA/ml, and stored −70 °C for future use.

2.4. RT-PCR for detection of chemokine mRNA

Spleen RNA (5 μg) was transformed to cDNA using oligo (dT) primers (0.5 μg/reaction) and reverse transcriptase (Super Script® II RT-Gibco, 200 U/reaction), in Tris/HCl 50 mM, 75 mM KCl, 3 mM $MgCl_2$ and 0.01M DTT, containing 1 mM of dNTPs, in a 25 μl final volume. The reaction was incubated at 37 °C for 1 h and immediately used or stored at −70 °C. PCR reaction 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/HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and 0.2 mM of 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 [22], were: SDF1-α (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 Tris–borate–EDTA 6% polyacrylamide gel electrophoresis with silver staining, using consecutive lanes for β-actin, SDF-1α and SDF-1β of each experimental time and animal. Dried gels were scanned and uncalibrated absorbance of specific bands quantified using a 10 × 20 pixels window, by the ImageJ free software [http://rsb.info.nih.gov/ij/]. Values were expressed as percent of β-actin mRNA, after background substraction, using the same β-actin RT-PCR product in the same reaction and scan as unit.

2.5. Supplementation of SDF-1α in P. berghei malaria

We analysed the effect of exogenous SDF-1α in groups of five BALB/c and C57BL/6 mice infected with 10^6 *P. berghei* pRBCs i.p., as described above, with daily determination of parasitemia. Exogenous SDF-1α (four doses, 1 μg/mice, i.p.) was injected on selected days, controls mice receiving sterile saline, and parasitemia and mortality were determined in treated and control mice groups until 20 days after infection. Mean values of parasitemia were compared by ANOVA, with Bonferroni post-tests after variance check, with a 0.05 significance level ($P < 0.05$).

2.6. T-140, SDF-1 receptor blocking peptide, effect on P. chabaudi malaria

Groups of five C57BL/6 mice were infected i.p. with 10^6 *P. chabaudi* pRBCs, as described above, and T-140 was administered twice daily, 10 μg/dose/mouse, injected i.p. on days 4–8 after infection, each mouse receiving a total dose of 100 μg. Infected non-treated mice were used as controls, with sham injection of sterile saline at same experimental time. Parasitemia were determined daily in all groups until day 20 after infection, and statistically compared as described above.

3. Results

3.1. Parasitemia and SDF-1 mRNA expression

As can be seen in Fig. 1A and B, *P. chabaudi* induced similar pRBC high levels. These were spontaneously controlled in both mouse strains, despite minor differences in peak numbers, which were more marked in susceptible BALB/c mice. The course of the disease was highly reproducible and mainly without mortality. Deaths were occasionally recorded among BALB/c mice (1/20), but not among resistant C57BL/6 mice, despite their peaks of parasitemia of over 50% after 10^6 pRBC infection. Host recovery was quick and resumed within 4 days after the peak at day 8 of infection; low (1–2%) recrudescence of parasitemia was observed in both mouse strains, occurring after day 14 of infection. The same reproducibility was found in *P. berghei* infection (Fig. 1C and D), where parasitemia appears at 3–5 days after challenge ip with 10^6 pRBC, with progressive increase until day 15 post-infection. At this time, 25% mortality was observed, with survivors presenting severe disease and deaths occurring until day 20 in all groups. There were minor mouse strain-related differences in the evolution of parasitemia, mostly regarding day 10, when a clear attempt to control blood parasite numbers was observed in *P. berghei* infected C57BL/6 mice (Fig. 1C).

We also analyzed the production of mRNA encoding for SDF-1α and -1β in pooled spleens of three animals of our experimental models, in parallel with the course of parasitemia. Digital imaging of scanned silver stained

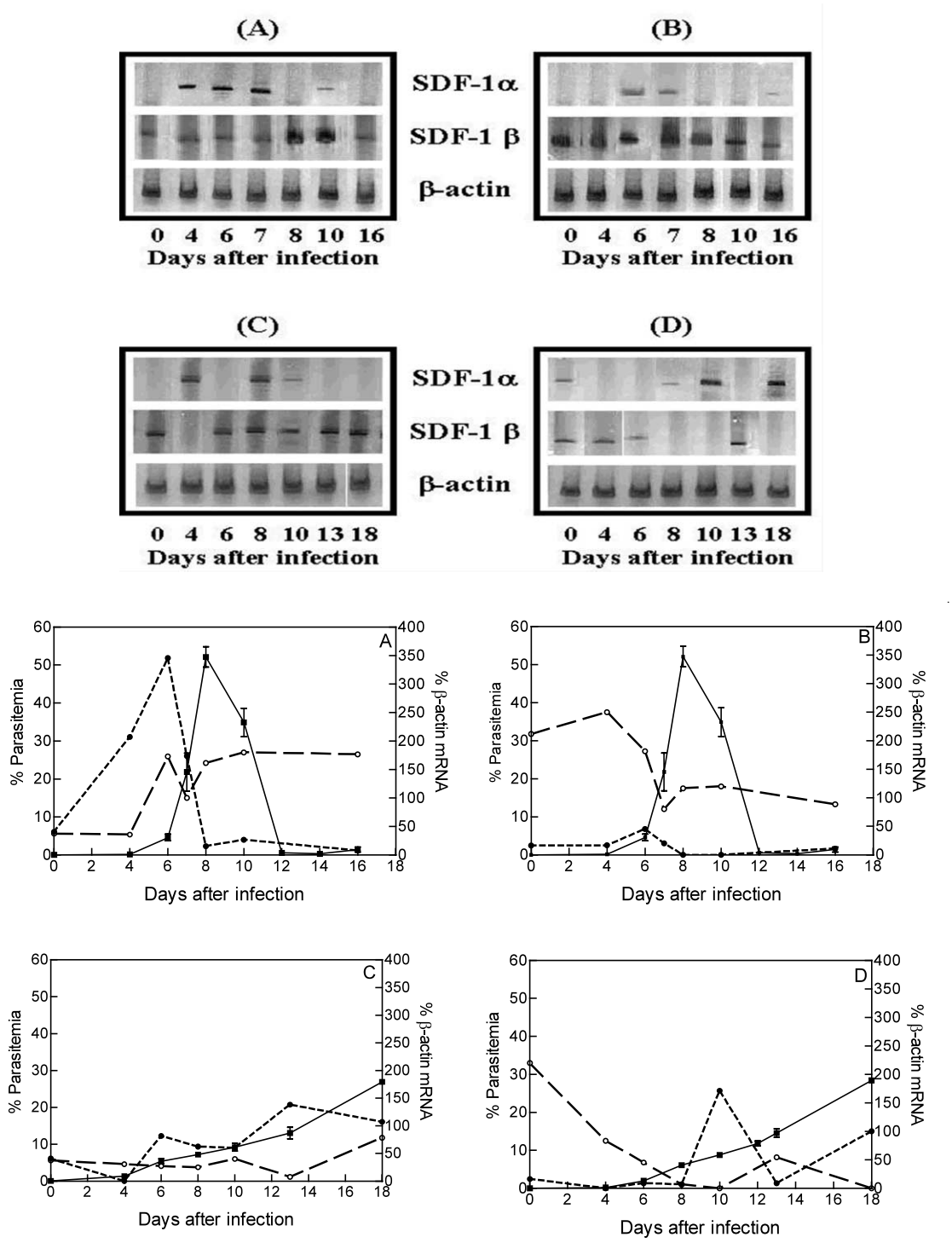

Fig. 1. Parasitemia and sequential production of SDF-1α and -1β mRNA by spleen cells in experimental malarias. Top: Digital imaging records of SDF-1 RT-PCR from pooled spleens cells from three mice in each point of one typical experiment. Bottom: parasitemia and semi quantitative mRNA quantification of this experiment, as described in Section 2. *P. chabaudi* infection in C57BL/6 mice (A) and BALB/c mice (B); *P. berghei* infection in C57BL/6 mice (C) and BALB/c mice (D). Bars represent SEM of parasitemia. Solid line: parasitemia. Dotted line: SDF-1α mRNA production of spleen cell, expressed as percent of β-actin mRNA. Dashed line: SDF-1β mRNA production by spleen cells, expressed as percent of β-actin mRNA.

gels of specific bands of a typical experiment could be seen at the top of the figure, allowing the quantification as stated in Section 2 and thereafter presented. The production of SDF-1α mRNA occurred mainly at the peak of ascending parasitemia in *P. chabaudi* infected mice (Fig. 1A and B) and declined or disappeared

thereafter, the peak being much more intense in C57BL/6 mice. SDF-1α mRNA expression in *P. berghei* infected mice increased slowly after day 6 of infection in C57BL/6 mice, with no detectable peaks (Fig. 1C). In BALB/c mice infected with *P. berghei*, there was only an occasional production of this chemokine at day 10 (Fig. 1D), a fact also confirmed in other experiments. Production of SDF-1β mRNA was present at all the experimental times and increased during the course of infection in C57BL/6 mice, but was also found sporadically in BALB/c mice. The production of this chemokine was higher in spleens of *P. chabaudi* infected mice as compared with *P. berghei* infected mice, despite the absence of peaked or parasitemia-related production. This data was reproducible in at least three experiments, but the variation on imaging quantification was higher avoiding statistical analysis between groups.

3.2. Supplementation of SDF-1α in P. berghei malaria

The evolution of parasitemia in groups of five chemokine-treated and control mice, performed as described in Section 2, could be seen using human recombinant (Fig. 2A and B) or mouse synthetic (Fig. 2C and D) SDF1-α. Treatment consisted of four doses, indicated by arrows, administered during the same period that peak SDF-1α mRNA expression was detected in *P. chabaudi* infection. There was a clear effect of chemokine treatment on parasitemia, which decreased after 2 days of treatment and thereafter, resulting in lower parasitemia at latter times. The experiment was stopped at 20th, and the survival at day 18th of all joined experiments showed 80% (16/20) for SDF-1 receiving mice and 50% (10/20) of infected controls, with a borderline significance, as determined by χ^2-test ($P = 0.05$).

3.3. SDF-1α receptor blocking by treatment with T-140: effect on P. chabaudi infection

We infected groups of C57BL/6 mice with 10^6 *P. chabaudi* pRBC i.p. and treated them with T-140 at ascending parasitemia, as described in Section 2. Mice receiving repeated doses of the peptide presented higher and earlier parasitemia ($P < 0.001$) as compared to non-treated infected controls; blocking of the SDF-1α receptor also facilitated recrudescence, with higher levels of parasitemia (Fig. 3). Initial lower undivided single dose trials failed to show any effect of T-140 on our models, showing that the peptide had a short-lived effect on the host (data not shown); the higher concentration was tested only in the best controlled mouse-malaria association, previously determined.

4. Discussion

Our experimental malaria models showed good reproducibility, similarly to data described elsewhere, and allowed a sequential comparison during infection

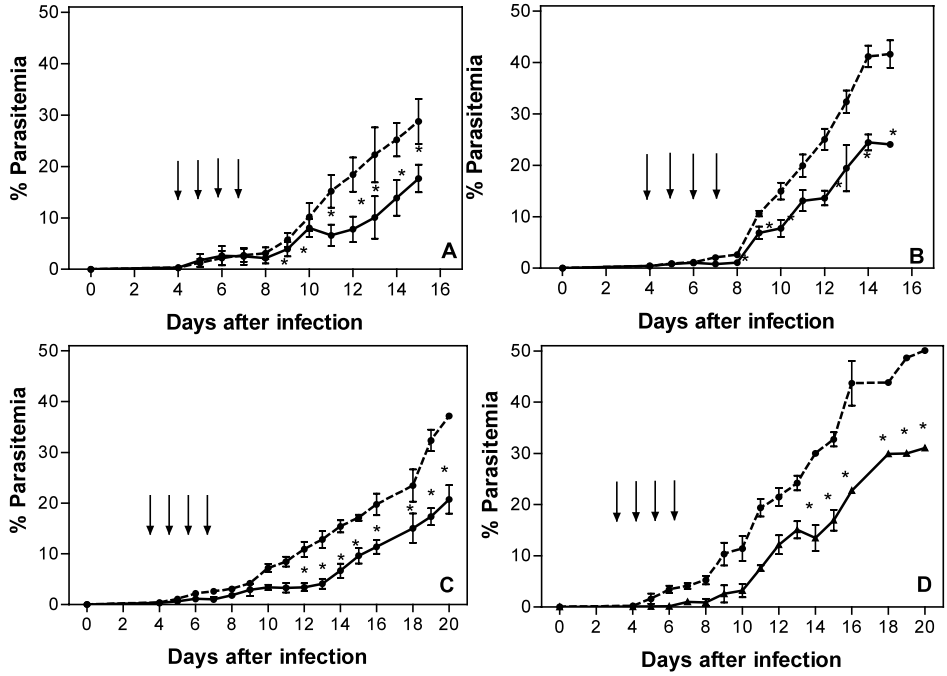

Fig. 2. Effects of supplementation with human recombinant (A, B) or mouse synthetic (C, D) SDF-1α during the *P. berghei* infection of groups of five C57Bl/C mice (A, C) or BALB/c mice (B, D). Bars represent the SEM of parasitemia. Arrows indicate the time and numbers of chemokine i.p. injection in experimental animals. Solid line: chemokine injected animals Dotted Line: control mice. Asterisk marks significant Bonferroni post-tests ($P < 0.05$) between chemokine treated and control mice.

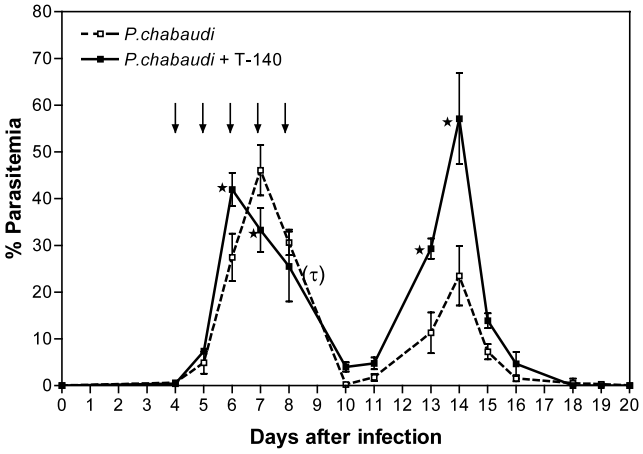

Fig. 3. Effects of CXCR4 blocking T-140 during *P. chabaudi* infection in groups of five C57BL/6 mice. Bars represent the SEM of parasitemia. Arrows indicate the time of CXCR4 blocking T140 ip injection in experimental animals. Solid line: injected animals. Dotted line: control mice. Asterisk marks significant Bonferroni post-tests ($P < 0.05$) between chemokine treated and control mice. τ marks the day of death of one receptor blocking injected animal.

[7,22,23]; our selection of parasite and mouse strains avoided models with less similarity to human disease [24]. The clearing function of the spleen is known to be similar in both our models, independently of the *Plasmodium* species, and is probably related to the enlargement of the splenic filtration network [7]. This is a critical point in experimental malaria, since the disease in man is multifaceted and rapidly modified by the immune response, which must be quickly established for an effective result, as otherwise seen in human cerebral disease, described both as cytoadherence parasite dependent [25] or as an immunopathological consequence of the infection [26].

Dendritic cell activation has been described in malaria [2], and our data support this concept, since our increased SDF-1 synthesis could be attributed to those cells, the main SDF-1 producing cell population at the spleen [8]. SDF-1α production mainly occurs at ascending parasitemia in *P. chabaudi* non-lethal malaria, suggesting the coordinate action of immune cells in the induction of protective immunity, which is said to be located in the spleen [2]. The increased production of SDF-1α presently described for non-lethal malaria could be driven by inducible synthesis or an increase in specific cell population, as most studies attribute a constitutive synthesis to this chemokine [27]. The absence of peak production in lethal models could be explained by early cytokine synthesis, especially of IL-1 and TNF-α, which have been described to have an inhibitory effect on SDF-1 production in mesenchymal cells [12]. SDF-1β production was present in most experimental times in both our models, suggesting that another than SDF-1α producing cell populations could be involved in their synthesis. SDF-1α production in *P. berghei* lethal malaria did not peak early in the infection and was not related to the evolution of parasitemia and frequently absent. The feeble attempt to control blood parasite numbers in this model, which occurred on day 10 of infection in C57BL/6 mice, also correlated with an increase in SDF-1α mRNA. Concurrently, SDF-1β production appears not as marked as that found in *P. chabaudi* infection. These data suggest that the immune response in the *P. berghei* model must be severely impaired in the early stages of infection, resulting in ineffective immune response and disease progression. As *P. berghei* schizont-infected cells adhere preferentially to erythroid areas, as the spleen red pulp [28], the local parasite growth could disturb the immune response, explaining the observed erratic SDF-1α production, an effect which was absent in non-lethal *P. chabaudi* malaria. Interestingly, nitric oxide mediated inhibition of dendritic cells has been reported [29]. Despite reports of increased spleen NO production in *P. chabaudi* infection [30], the data on its function in malaria are conflicting [31].

SDF-1α and -1β perform mainly two functions. One reflects a haematopoietic role and relates to stem cell homing and proliferation [17]; the other is immunologic and relates to the amplification of a cellular immune response, especially of T and B lymphocyte subsets [32]. Our data suggest that these chemokines may interact in diverse environments with specific cell subsets and result in one or more main effects. The SDF-1 actions on organogenesis, demonstrated by knockout studies [11], also illustrate the critical significance of these chemokines on primordial steps of cell function, when they probably organize the early environments of effective immune responses [4]. Stromal derived factor 1 is genetically conserved, without differences between human and mice protein sequences [33], and our observations lend support to this, as we obtained similar results using human and mouse chemokines. One simple explanation for the exogenous SDF-1 induced decrease in parasitemia could be a dilution effect due to massive expansion of blood cell populations. However, the observed drop in blood parasites was too intense to be accounted for by this hematopoietic effect alone. A more effective immune cell homing to the white pulp could be an additional effect, possibly resulting in a better immune response against pRBC by affecting receptor presentation or active cell recruitment, as the targets of SDF-1 are CD34+ cells, usually antigen presenting or macrophagic cells [9].

The main SDF-1α producing cell population is likely to be blocked or destroyed during the early stages of *P. berghei* infection, as also suggested by others [12]; supplementation with the synthetic or recombinant chemokine could hence partly overcome the absence of production in the infected host, resulting in correction of cell homing and proliferation and increased parasite

clearance. A systemic effect on antibody and myeloid responses, however, cannot be excluded [10,11]. The effect of CXCR4 blocking with T-140 in non-lethal malaria must be related to blocking crucial and adequate cell homing, since, after its removal, the mice were unable to completely mount effective immunity. Inhibition of the CXCR4 receptor activation affects all the SDF-1 actions, thus it was not surprising to see a complex effect on the immune response in our mouse models. Beside to this, two distinct dendritic cell subsets were described in the spleen, by cell surface antigen expression and area distribution, with myeloid or lymphoid specific functions [34]. Structure related and isolated cell SDF-1α production must be determined to clarify their effect in immune responses to malaria in the spleen.

Primordial chemokines like SDF-1, usually related to a supportive care of the immune and others host responses; would be an ideal target for agents capable of interfering with molecular function/expansion of chemokine producing cell populations or of inducing high NO production, with subsequent apoptotic death of producing cells [30]. Our data suggest that SDF-1α production in the spleen is important for the establishment of parasite clearance in rodent malaria, and its supplementation might partially correct defects in the control of lethal malaria. Hypothetically, supplementation with primordial chemokines could correct some defects of the immune response observable in selected experimental models or in patients.

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