Supplementation of CXCL12 (CXCL12) induces homing of CD11c+ dendritic cells to the spleen and enhances control of \textit{Plasmodium berghei} malaria in BALB/c mice

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Summary

In malaria, parasitaemia is controlled in the spleen, a multicomponent organ that undergoes changes in its cellular constituents to control the parasite. During this process, dendritic cells (DCs) orchestrate the positioning of effector cells in a timely manner for optimal parasite clearance. We have recently demonstrated that CXCL12 [stromal cell-derived factor-1 (CXCL12)] supplementation partially restores the ability to control parasitaemia in \textit{Plasmodium berghei}-infected mice. In the present study, we investigated the nature of the DCs involved by flow cytometry and immunohistochemistry of CD11c+ cells. Flow cytometry of bone marrow cells showed that infection with \textit{P. berghei} did not alter the proportion of CD11c+ cells present in this haematopoietic compartment, while CXCL12 supplementation of naïve uninfected mice induced only minor increases in the population of CD11c+ cells. In the spleen, \textit{P. berghei} infection alone resulted in an increase in CD11c+ cells as compared with naïve animals. Exogenously administered CXCL12 in the absence of infection resulted in a significant expansion of the splenic CD11c+ population, and this effect was even more pronounced in infected and supplemented mice. Immunohistochemistry revealed that CD11c+ cells infiltrated the perivascular areas and marginal zone of the spleen in infected animals treated with CXCL12, suggesting that this chemokine induces homing of CD11c+ dendritic cells to the splenic compartment. Our results show that small amounts of CXCL12 supplementation are effective in recruiting DCs to the spleens of both uninfected and infected mice, suggesting the participation of CXCL12 and CD11c+ cells in the establishment of an adequate environment in the spleen for malaria control.

Keywords: \textit{Plasmodium berghei}; CXCL-12; spleen; CD11c+; dendritic cells; malaria

Introduction

Malaria is caused by infection of red blood cells with \textit{Plasmodium} species, and its control is in large part dependent on the spleen.1 The spleen, a multicomponent organ, is the clearance site for senescent or otherwise damaged red blood cells. It is also an important site for activation of the immune system, and it may serve as a blood reserve. During malaria, there is a significant organized expansion of splenic compartments, especially the red pulp but also the white pulp.2 The reticular, stromal and dendritic cells (DCs) of the spleen display several phenotypes and biological functions, most of them related to spatial allocation of cells.3 Splenic expansion depends on the migration of uncommitted cells to the spleen, probably from the bone marrow.4 Candidate cells are the primitive CD1a+ CD34+ or committed CD11c+ cells, from the stem cell dendritic lineage.5 The engagement of DCs in the earliest phase of malaria infection in vivo has been described in an experimental rodent malaria model by Leisewitz \textit{et al.}, who postulated a crucial role of DCs in the shaping of the immune response, although they did not address DC homing.4 The fate of DCs in the spleen is at present disputed and controversial, but they may be sorted into three defined subsets according to their expression of CD4 and CD8: CD11c+ CD4+ CD8– make up around 20% of DCs, CD11c+ CD4– CD8+ 60%, and CD11c+ CD4– CD8– 20%.\textsuperscript{5,6} CD11c+ CD8α– DCs are
located in the marginal-zone bridging channels and throughout the marginal zone into the red pulp, whereas CD11c+ CD8α+ DCs appear as interdigitating DCs in the T-cell zone of the white pulp. The putative functions of CD8α+ DCs have been postulated to relate to the presentation of soluble antigens to CD4+ T cells, whereas CD8α+ DCs present cell-associated antigens. Immature DCs express CCR1, CCR2, CCR5 and CXCR1, which guide them to inflammatory sites for maturation and antigen sampling. Other chemokine receptors, such as CCR7, CCR9, CXCR4 and CX3R1, are also found in different subsets of DCs. The DC maturation process, which may be triggered by inflammatory cytokines and bacterial or viral products, leads to down-regulation of receptors for inflammatory chemokines and up-regulation of receptors for constitutive chemokines such as CXCR4, CXCR4 and CCR7. These latter receptors guide the maturing DCs in their migration to and maintenance of the microanatomic environment of secondary lymphoid organs, such as the spleen.

Chemokines mediate several cellular functions in addition to migration, such as maturation, proliferation, survival, effector responses and organogenesis of some non-lymphoid structures such as the heart and the cerebellum. They include more than 50 chemokines and 18 chemokine receptors. CXCL12 [or stromal cell-derived factor-1 (SDF-1)] and its unique receptor, CXCR4, enhance the proliferation and survival of haematopoietic stem cells in association with other cytokines, and inhibit the effects of myelosuppressive chemokines. In addition to being a potent chemotactic factor for T and pre-B lymphocytes and DCs, CXCL12 also has an effect on T-cell rolling and tight adhesion to activated endothelial cells, and on haematopoietic cell homing. CXCL12 is produced and secreted by several types of stromal cells of the bone marrow, mainly immature osteoblasts. It is expressed by endothelial cells and is involved in the modulation of the microenvironment for homing of precursor cells.

We have demonstrated that in non-lethal rodent malaria there is a synchronous production of CXCL12 during parasitaemia control, and that this production is erratic in lethal malaria, when mice are unable to control parasitaemia. Furthermore, when CXCL12 was given to mice with lethal malaria, parasitaemia was partially controlled, while T-140, a competitor of CXCL12, worsened the disease in non-lethal malaria. We have also reported that CXCL12 production is affected by nitric oxide and interferon (IFN)-γ. CXCL12 synthesis could be associated with DC migration to the spleen during experimental malaria. By participating in DC homing, this chemokine might thus be involved in the amelioration of disease and a decrease in parasitaemia. In this work, we investigated the effect of exogenous CXCL12 treatment on Plasmodium berghei malaria, and evaluated the percentage and distribution of DCs in the spleen and bone marrow by flow cytometry and immunohistochemistry.

Materials and methods

Parasite, animals and reagents

Female 6–8-week-old BALB/c mice from our colonies were used in all the experiments and were handled in accordance with the Guide for the Care and Use of Laboratory Animals. Plasmodium strains were stored as stabilates in liquid nitrogen. Monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (BD PharMingen, San Diego, CA) were used for CD11c, CD11b and CD8α typing, with adequate isotype controls. The source of each specific reagent is given in the text.

Experimental malaria models

Stabilates containing parasitized red blood cells (pRBC) were injected into naive mice a week before the experiments began. Mice were injected intraperitoneally (i.p.) with 10^6 pRBC for reproducible infection. Parasitaemia was monitored daily using Giemsa-stained thin tail blood smears. The spleen and bone marrow were aseptically removed and immediately processed for flow cytometry. Cells from the spleen were mechanically dissociated in 5 ml of Dulbecco’s modified Eagle’s minimal essential medium (DMEM) with 5% fetal calf serum (FCS) and antibiotics, with incubation with clostripain-free collagenase IV from Clostridium perfringens (Sigma, St. Louis, MO) for 30 min at 37°C. For each experiment, groups of at least three animals were used. For histology, spleens were immediately fixed with 4% p-formaldehyde in cold phosphate-buffered saline (PBS).

Exogenous CXCL12 in P. berghei malaria

P. berghei-infected BALB/c mice received exogenous synthetic mouse CXCL12 i.p. according to several dosing regimes; a group of saline-injected mice was included as a control. The intermediate doses of CXCL12 used (50 μg/kg or 1 μg/20 g mouse) were based on established protocols for radiation haematological rescue in mice. Parasitaemia was determined at daily intervals in treated and control mice until day 16 of infection. Mean values were compared by analysis of variance (ANOVA), with a Bonferroni post-test after variance check; the level of significance was 0.05 (P < 0.05).

Flow cytometry

Cell-surface immunophenotypic analysis was performed by flow cytometry. Bone marrow cells or collagenase-
treated spleen cell suspensions were recovered by centrifugation at 200 g for 10 min at 4 °C, and washed twice in PBS containing 5% FBS, with subsequent Fc blocking treatment for 30 min at 4 °C. After additional washing, optimal concentrations of specific primary Abs were added and the cells were incubated for 30 min at 4 °C in PBS containing 5% FBS. Cells were washed and fixed in 0.5% paraformaldehyde. At least 10 x 10^4 cells were used for each organ or animal. Samples were analysed by forward and side light-scattering parameters (FSC and SSC) with gating for large and granulose cells, using specific antibody fluorescence on a FACScalibur (Becton and Dickinson, Mountain View, CA).

Histology

Spleen fragments from each animal were fixed in 20 volumes of 4% p-formaldehyde in 0.05 M NaPO₄, pH 7.2, for at least 4 hr, and routinely processed for paraffin embedding. Tissue sections (4 μm thick) were adhered to sylane-treated slides. The sections were rehydrated and deplated of endogenous peroxidase activity and hematoxylin. Non-specific binding sites were blocked with 10% defatted dry milk and biotin blocking reagent (DAKO, Glostrup, Denmark). Antigen was exposed by microwave treatment and the sections were incubated with hamster immunoglobulin G (IgG) antimouse CD11c in PBS containing 1% bovine serum albumin (BSA). After washing, the slides were treated with secondary biotin-labelled rabbit antihamster IgG for 1 hr and then incubated with avidin-biotin horseradish peroxidase complex. Immune complexes were visualized by incubation with 0.01% H₂O₂ and 0.05% 3,3′-diaminobenzidine tetrachloride (DAB). Sections were counter-stained with Harris haematoxylin. Representative fields were recorded using a Leika microscope with a digital camera (Pixera, Los Gatos, CA).

Results

Dose–response effect of supplementation of CXCL12 in P. berghei malaria

Groups of five BALB/c mice were infected with P. berghei as described in ‘Materials and methods’. To investigate the dose–response effect of CXCL12 supplementation, each group was injected i.p. with CXCL12 according to several protocols. In addition to our main approach of four daily injections of 1 μg (50 μg/kg) from day 4 to day 7 of infection, another group received 11 daily injections of 1 μg of CXCL12 starting on day 4 until the end of the experiment (day 16). Two other groups received four daily injections of 0.2 μg (10 μg/kg) or 5 μg (250 μg/kg) of CXCL12 from day 4 to day 7 of infection. The results presented in Fig. 1 show that exogenous CXCL12 reduced parasitaemia in all the treated groups. However, this effect appeared to be dependent on the first 4 days of chemokine administration, because further CXCL12 supplementation beyond day 8 did not induce progressive parasitaemia control. We did not observe a typical dose–response effect. The doses of chemokine tested are likely to fit the plateau range, suggesting a very low threshold.

Effect of rodent malaria and CXCL12 on the proportion of DCs in the bone marrow

Groups of mice infected with P. berghei and supplemented or not with 1 μg/day of CXCL12 from the 4th to the 7th day of infection were killed at day 14. Bone marrow cells were submitted to CD11c phenotyping, and subtyping with CD11b or CD8α markers, by flow cytometry. As shown in Fig. 2, CXCL12 supplementation of infected mice resulted in a very slight and non-significant increase in bone marrow CD11c+ cells and no changes in the subpopulations. In naïve mice, exogenous CXCL12 led to a small but significant increase in bone marrow CD11c+ cells with no differences in subtypes CD11b+ or CD8α+.

Effect of rodent malaria and CXCL12 supplementation on the proportion of DCs in the spleen

Groups of naïve and P. berghei-infected mice treated or not with four doses of 1 μg of CXCL12 from the 4th to the 7th day post-infection were killed on day 14. Spleen cells were submitted to CD11c phenotyping, with CD11b and CD8α subtyping. Non-infected mice were similarly treated with CXCL12 and also killed on day 14. The
proportions of CD11c+ CD11b+ and CD11c+ CD8α+ cells were slightly enhanced in infected mice as compared with naive mice (Figs 3a and b). Exogenous CXCL12 in the absence of infection similarly enhanced the two subtype populations. In non-infected animals, treatment with CXCL12 increased only the CD11c+ CD11b+ population, with no changes in CD11c+ CD8α+ cells when compared with naive mice. There were greater variations in cell proportions after infection, which affects the comparision of subpopulations in those groups. Typical cytometric profiles of each subpopulation are shown in Fig. 3(c).

Immunohistochemistry for CD11c in the spleen during rodent malaria and CXCL12 supplementation

Spleen samples from all mice groups were submitted to paraffin embedding, sectioning and immunohistochemistry with anti-CD11c antibodies, with strict internal controls. Representative fields of the spleen are shown in Fig. 4. Spleens from naive mice presented delicate staining of CD11c+ cells in the marginal areas, in a random scattered pattern, showing a typical low nucleated cellularity in the red pulp. Malaria induced a significant increase in CD11c+ cells, with large clusters of them around arterioles and marginal sinuses, as seen in Fig. 4(b). We observed disorganization of the red pulp with increased nucleated cell content, and several groups or clusters of cells likely to be proliferating (mainly as a result of extramedullary erythropoiesis) that were negative for CD11c staining. We also observed pigment deposition indicating parasite clearance. Supplementation with CXCL12 alone induced clear peritheriolar CD11c clustering, as shown in Fig. 4(c), which had the appearance of consisting of recently arrived non-activated cells. The spleens of infected and CXCL12-supplemented mice revealed a mixture of the patterns found for infection alone or CXCL12 supplementation alone. As shown in Fig. 4(d), there was more pronounced staining of the red pulp and marginal areas, as well as a clear peritheriolar staining of cell clusters, indicating an increased proportion of positive cells in the spleen and confirming that CXCL12 supplementation induced homing of CD11c+ cells to that organ.

Discussion

In this study, we clearly showed a protective effect of CXCL12 supplementation in a lethal rodent malaria model. In a previous investigation of non-lethal self-controlled malaria in mice, we detected, by reverse transcription–polymerase chain reaction (RT-PCR), specific early spleen production of CXCL12 that was directly related to the ability of infected animals to control parasitaemia.19 This result supports our present finding of exogenous CXCL12-induced partial protection in the early stages of a primary infection by P. berghei. The lower dose of CXCL12 used (0-2 μg/mouse or 10 μg/kg) caused a reduction in parasitaemia similar to that observed with the higher doses. Our data probably reflect a very low threshold for the chemokine effect, with the doses tested already yielding a maximal response plateau. We selected our dose range on the basis of the CXCL12 supplementation usually employed for radiation haematological rescue,22 which apparently was too high for determining a dose–response curve in our malaria model.

The effect of CXCL12 on P. berghei infection observed in the present study could be related to the response of specific cells that make up a discrete population in the spleen, possibly the DCs, which are found to increase in number in the splenic compartment in non-lethal Plasmodium chabaudi malaria.4 This increase correlating with an increase in the splenic production of CXCL12.19 Despite the observed effect on CD11c+ homing of exogenous CXCL12 in the spleen, other cell populations could also be involved in this process, such as those also expressing the CXCR4 receptor, for example haematopoietic or lymphoid lineages.14,15

In our present model, we showed that the splenic CD11c+ dendritic cell population was slightly increased by P. berghei infection. Exogenous CXCL12 treatment induced a rise in the CD11c+ CD11b+ and CD11c+ CD8α+ subsets in infected animals. This could be explained by earlier descriptions of the effect of CXCL12 on the mobilization and homing of haematopoietic and progenitor cells from the bone marrow to the periphery.18 In bone marrow, we showed that CXCL12 did not alter the percentages of DCs, especially the CD11c+ CD11b+...
subtype. There are reports indicating that circulating toxins or other products could interfere with blood-forming cells in the bone marrow.23 Although it could be speculated that this might apply to the present malaria model because it presents schizogony in the period investigated,19 we did not find major effects on the percentages of bone marrow CD11c+ subsets. Bone marrow cells were only estimated as percentages, because it is not possible to infer their exact numbers in this compartment, as a consequence of the absence of exact total cell counts or volumes.24 However, the fact that there were no major modifications in the observed percentages suggests that the main effect of CXCL12 occurs outside the bone marrow.

Immunohistochemistry showed a significant increase in the splenic CD11c+ population of infected mice as compared with naive animals. This difference was less pronounced when the organ was analysed by flow cytometry, probably because malarial spleens also presented large numbers of small, round stained cells that were excluded when a large granulose cell gate was applied. Furthermore, when processing cells for flow cytometry, a dilution effect from the very pronounced congestion of the spleen during malaria should also be taken into account. Immunohistochemistry may reveal a picture closer to the true in vivo splenic compartmentalization in malaria infection.

Recently, it was shown that splenic DCs defined by the CD11c marker migrated into the CD4+ T-cell area and

![Graph](image-url)
were actively engaged in *P. chabaudi* infection. In our present model, the flow cytometry data suggest that infection with *P. berghei* expands CD11c⁺ CD8α⁺ cells preferentially. Exogenous CXCL12 administered to infected mice increased both populations, but the CD11c⁺ CD8α⁺ subset seemed to predominate over CD11c⁺ CD11b⁺ cells. A previous phenotypic analysis of DCs in the spleens of BALB/c and C57Bl/6 mice with *P. chabaudi* infection showed that there are two major CD11c⁺ subpopulations, according to the expression of the CD8α receptor: CD11c⁺ CD8α⁺ cells make up approximately 60% of the CD11c⁺ cells and CD11c⁺ CD8α⁻ approximately 40%, the latter being highly positive for CD11b. Such data are in agreement with our findings on the expansion of the CD11c⁺ CD8α⁺ subset after CXCL12 supplementation in *P. berghei*-infected mice. However, mobilization of DC progenitors as a result of exogenous CXCL12 treatment could also recruit CD11c⁺ CD11b⁺ cells, and that might explain the expansion of this population in infected and treated animals as compared with the other groups. We have not looked thoroughly at the activation markers of these DCs, but our preliminary data on the expression of the CD40 seem to suggest that CXCL12-recruited cells maintain the same level of expression of this marker (data not shown), without a specific effect on cell activation.

The function of CD11c⁺ DCs is usually related to the early steps of an immune response, and this population was found to be affected in our rodent malaria model. Other authors have also proposed dysfunction of reticular cells in the spleen in such models. This effect could be explained by several reported aspects of malaria infection, the first being the processing of huge amounts of antigen for presentation, which might result in the commitment of a large proportion of a small, key cell population that consequently became unavailable for other kinds of immune cell co-operation. The loss of cells that produce CXCL12, a chemokine that promotes homing of CD11c⁺ DCs, might also result from early cytokine or free radical production, as we have previously shown a negative correlation between nitric oxide and IFN-γ production and CXCL12 synthesis in rodent malaria. This latter effect is probably caused by the induction of apoptosis of CXCL12-secreting cell populations, as apoptosis is a frequent event in both spleen and bone marrow during the immune response in malaria. The selection of specific cell types is a common immunological event in malaria, with high production of regulatory cytokines, such as interleukin (IL)-12, and also of chemokines. Another possible explanation is a direct effect of the parasite on such cell populations, possibly via exotoxins or other products, such as those described for haematopoiesis.

CD11c⁺ cells express CXCR4, the unique receptor for CXCL12, and we found increased homing of these cells in the spleen during CXCL12 supplementation of *P. berghei*-infected mice. The homing of these cells is probably dependent on CXCL12, which is insufficient or asynchronous in lethal malaria infection, as we have previously shown. This chemokine is responsible for the homing of several primitive cells, for example DCs and stem cells, and its availability must be perfectly orchestrated in the spleen in order to provide the correct organization of the organ and an adequate immune response. There are other participants in this orchestration, as we must remember that there are several different alleles for the CXCL12 receptor, CXCR4, a situation that may be compared with that described for HIV receptors in an African population.
It may be speculated that the CXCL12–CXCR4 receptor axis could strongly influence the establishment of severe malaria, either as severe anaemia or as multiorgan involvement.

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References

12 McDevitt MA, Xie J, Gordeuk V, Kwon HJ. Rapid and efficient homing of human CD8alpha(–) and CD8alpha(+) dendritic cells are generated from CD4(low) lymphoid-committed pre-lineages revisited: both CD8alpha(–) and CD8alpha(+) dendritic cell lineages. Blood 2004; 100:47–53.


